BIOCHEMISTRY OF PHENOLIC COMPOUNDS IN WHEAT GRAIN (*Triticum aestivum* L.)

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ABBREVIATIONS

***O*-DPO**	*o*-Diphenol Oxidase
POD	Peroxidase
MHQ	2-Methoxyhydroquinone (2-methoxy-1,4-dihydroxybenzene)
Cv(s)	Cultivar(s)
PAL	L-Phenylalanine Ammonia-Lyase
CHI	Chalcone-Flavanone Isomerase
CHS	Chalcone Synthetase
P.E.	Post-Emergence (after emergence of wheat ear from flag leaf)
TLC	Thin-Layer Chromatography
CIMMYT	International Centre for Maize and Wheat Improvement, Mexico.
PC	Paper Chromatography
HPLC	High-Performance Liquid Chromatography
C-18	Octadecylsilica
TFA	Trifluoroacetic acid
TCA	Trichloroacetic acid
L-Phe	L-Phenylalanine
PVPP	Polyvinylpyrrolidone
ANOVA	Analysis of Variance
N.S.	Not Significant at 5% Rejection Level
SEM	Standard Error of the Mean

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ABSTRACT

Phenolic content, *o*-diphenol oxidase activity, flour colour and bread crumb colour have been examined for flour streams from a commercial flour mill and in flours and wholemeals of a number of New Zealand wheat cultivars. Phenolic content and *o*-diphenol oxidase activity varied significantly both between milling flour streams and between cultivars. Flour and bread crumb colour correlated significantly with phenolic content and *o*-diphenol oxidase activity in the milling flour streams. A similar relationship was also observed between *o*-diphenol oxidase activity and the colour of flour and bread prepared from several different cultivars. These observations are consistent with an involvement of *o*-diphenol oxidase and phenolics in the pigmentation of both wheat flour and bread.

Commercial wheat bran has been found to contain low levels (<50 $\mu g.g^{-1}$) of (+)catechin and soluble proanthocyanidins soluble in aqueous acetone. Detection of these compounds in crude extracts by conventional methods was complicated by the presence of methoxyhydroquinone glycosides and other interfering substances. Chromatographic studies indicated that, in addition to several dimeric proanthocyanidins, bran contains trimeric and/or other oligomeric proanthocyanidins. The oligomeric proanthocyanidins contained mostly prodelphinidin and some procyanidin units, whilst the dimeric proanthocyanidins may also contain some propelargonidin units. Two dimeric proanthocyanidins were isolated and tentatively characterized as catechin-(4$\alpha\rightarrow$8)-catechin (procyanidin B3) and gallocatechin-(4$\alpha\rightarrow$8)-catechin (prodelphinidin B3). These observations suggest that the flavanol content of wheat grain may may be qualitatively similar to that of barley, although quantitatively much smaller. The identification of these compounds in mature wheat grain confirmed earlier reports of their presence in immature grain and supports the hypothesis that they contribute to seedcoat pigmentation.

Changes in soluble phenolics and hydroxycinnamic acids and the activities of L-phenylalanine ammonia-lyase (PAL, EC No. 4.3.1.5) and chalcone-flavanone isomerase (CHI E.C. 5.5.1.6) were examined during the development and maturation of several spring bread wheat cultivars.

Activities of both PAL and CHI were high in the early milk stage of grain development and this activity was located principally in the testa and pericarp tissues. Comparison of enzyme activities at this stage indicated significant differences between red- and white-grained cultivars. These different patterns of activity also correlated

with significant diferences in germinability at maturity. It is suggested that the common association between red seedcoat pigmentation and dormancy may be related to differences in the regulation of phenolic biosynthesis in the immature seedcoat. Studies of the tissue distribution of enzyme activities suggested that phenolic biosynthetic activities were higher in the embryo during the dough stage of grain development.

Chromatographic studies suggested that there were significant qualitative and quantitative changes in phenolic content during grain development and maturation. Changes in ferulic and other hydroxycinnamic acids indicated that these were subject to considerable turnover and metabolism during grain development.

SECTION 1-INTRODUCTION

1.1 Plant Phenolics and Their Biological Significance

Phenolic compounds, often referred to by the generic term "phenolics", are a diverse and abundant group of naturally occurring plant substances. They are characterised by the possession of a hydoxylated aromatic nucleus and thus the term "phenolic" embraces several major classes of compound as well as occasional members of other groups that contain phenolic units. Most phenolic nuclei are derived biosynthetically from 5-dehydroquinate via the shikimic acid pathway or from acetate via polyketide metabolism[1]. The most abundant phenols, in terms of biomass, are those derived subsequently from the pathways of phenylpropanoid and flavonoid biosynthesis.

The biological significance of phenolic compounds can largely be related to their characteristic chemical properties and reactivity. They are generally present in the cell as glycosides or esters and are thus fairly polar. All phenolic substances show strong absorption in the UV and many also absorb strongly in the visible region of the spectrum. Resonance effects confer stability to the phenoxide ion and to phenoxy radicals.

Phenolics are usually classified as "secondary" metabolites, suggesting they are of secondary importance, and such classification has tended to reflect and to perpetuate a lack of understanding of their physiological and ecological significance[2]. The idea that they are waste products or byproducts has lost favour and a wide variety of biological activities and functions have now been attributed to them. The most important of these include pigmentation, activity as protective agents, contribution to cell wall structure and as regulators of growth and development.

Much of the pigmentation of plant parts other than that due to chlorophyll and carotenoid pigments is due to phenolic pigments or to their oxidized, complexed, or otherwise modified derivatives. There is much evidence to suggest that phenolic compounds may play an important role in the adaptation of land plants to the associated problems of UV light and oxygen toxicity[3]. Some light mediated plant growth phenomena such as phototropism may be mediated through phenolic photoreceptors[4].

Numerous effects of phenolics on plant growth, development and metabolism have been reported and they are generally considered to be inhibitors[5]. This is an oversimplification, for although inhibitory effects of phenolics on biochemical or physiological processes are common, promotory and synergistic effects have been

frequently reported. Phenolics may affect plant metabolism through a variety of mechanisms. Many phenolics inhibit enzyme activities in a specific or non-specific manner, notably oxidative phosphorylation, ATPases and membrane transport processes[6]. In recent years evidence has been presented that they may directly influence gene expression[7,17]. Phenols have recently been shown to act as inhibitors of calmodulin-mediated protein phosphorylation[8]. Interactions of phenols with plant growth regulators have been reported in numerous systems but it is still uncertain whether endogenous phenols have a significant influence on plant development[5].

It is now widely accepted that many plant phenolics play an important role as protective agents against animals and microorganisms[2,9]. Toxic and inhibitory effects of phenols on cellular processes have also been observed in these organisms. Many polyphenols, notably condensed and hydrolysable tannins, markedly decrease the palatability and digestibility of plant material. Infection of plant tissue by pathogenic organisms is typically associated with qualitative and quantitative changes in phenolic content. Disease resistance has in some in some cases been related to phenolic content or to synthesis of phenolics in response to infection[10].

Like many other cellular components, phenolics have been shown to be subject to metabolic turnover in plant tissues. Plant metabolism greatly modifies the chemical properties of phenols by glycosylation, acylation,polymerisation, *o*-methylation, hydroxylation and oxidation[11]. Many biological activities and functions of phenolics are a consequence of their tendency towards spontaeneous or enzymic oxidation. Polymerization of phenoxy radicals generated through the activity of oxidative enzymes produce lignin and plant pseudomelanins[12]. Oxidative cross-linking of bound phenolic acids may influence the structure and extensibility of the cell wall[13]. Many phenolics only exhibit toxic or inhibitory properties after oxidation to the reactive quinone form. Oxidized phenolics show greatly enhanced biological activity, including acceleration of IAA oxidation[14] and inactivation of enzymes[15]. The characteristic darkening of cut or damaged plant tissues is a result of condensation of quinones, formed by enzymic oxidation of phenolics, with amino acids or proteins[16]. Such enzymic browning complicates extraction of plant enzymes and may serve as defense against pathogens.

In addition to their role as protective agents, phenolics released by leaching or degradation of plant material may have ecological significance as "allelochemicals" and as modifiers of soil chemistry[152].

1.2 Phenolic Compounds in Wheat Grain

The phenolic composition of wheat grain is qualitatively similar to that of other cereals seeds and tissues. Generally, it has been found that wheat contains somewhat

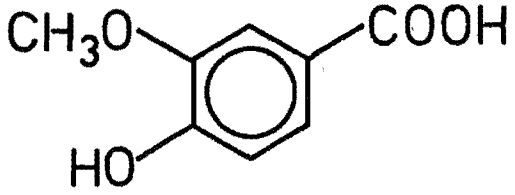
lower concentrations of phenolics compared to other cereal grains such as barley, oats,sorghum, millet and rye[18]. The bulk of the phenolics are concentrated in the germ (embryo and scutellum), with lesser amounts in the bran (testa, pericarp and aleurone) and only traces in the flour (endosperm)[19] (fig. 1.2).

The most abundant simple phenolics in cereals are the hydroxybenzoic and hydroxycinnamic acids (fig. 1.1). Early work[20] indicated that wheat grain contains a variety of phenolic acids including ferulic, *p*-coumaric, sinapic,syringic, *p*-hydroxybenzoic and vanillic acids. Although these were detected in trace quantities as the free acids they were found to be present mainly as soluble and insoluble conjugates. Later workers[21,22] used more sophisticated GC methods to identify and quantify these and other simple phenolic acids in flour hydrolysates but did not examine the nature of the conjugates themselves. Much work in recent years has shown that esterification of phenolic acids to cell wall polymers is characteristic of grasses[23]. However there is still little information concerning the structures of soluble conjugates of phenolic acids, although esters with triterpene alcohols[24,25] and flavones[26,27] have been reported.

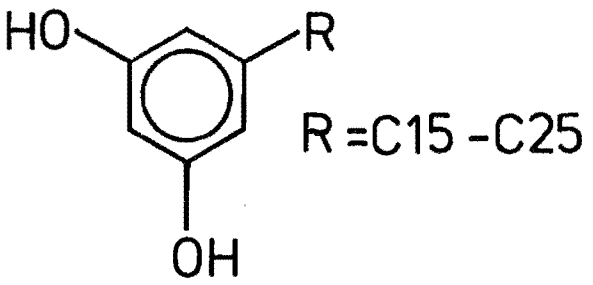
Another important group of simple phenols present in the mature wheat grain are the methoxyhydroquinone glucosides. These are among the most abundant soluble phenols in the mature grain and have been shown to have significance as anti-fungal agents[28] and for their effects on doughs[29]. They are concentrated mainly in the germ although lesser amounts may be present in the bran[30]. Mono-, di-,tri- and tetraglucosides have been reported to occur in commercial wheat germ[31,32]. Recent evidence[33] suggests that the triglucoside is the most abundant and the lower glucosides may be produced as a result of β-glucosidase action.

Flavones, in particular the C-glycosyl-flavones, are the most common type of flavonoid found in grasses. The first flavone to be characterised in wheat was tricin, which was identified as a rust resistance factor in wheat leaves[34]. Early work showed these compounds were also present in wheat grain[35] and subsequent studies by King[26] showed that the flavones in wheat germ were principally apigenin C-glycosides and their sinapyl esters. More detailed chromatographic and structural studies[27] indicated that germ contains a number of 6,8-di-C-glycosyl-apigenin derivatives and their isomers, as well as their sinapic and ferulic acid esters.

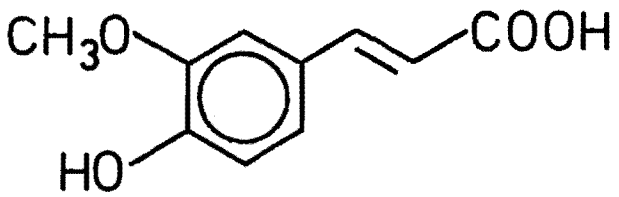
The germ, or embryo is a comparatively rich source of flavonoids, but little attention has been paid to the bran. Recent work suggests that bran also contains alkali-labile forms of di-C-glycosyl-apigenin derivatives[36]. The pigmentation of purple wheats is due to the presence of anthocyanins in the pericarp - principally cyanidin and



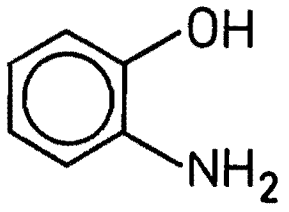
Vanillic Acid



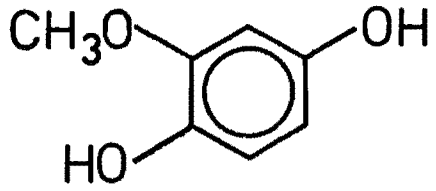
n-ALKYLRESORCINOL



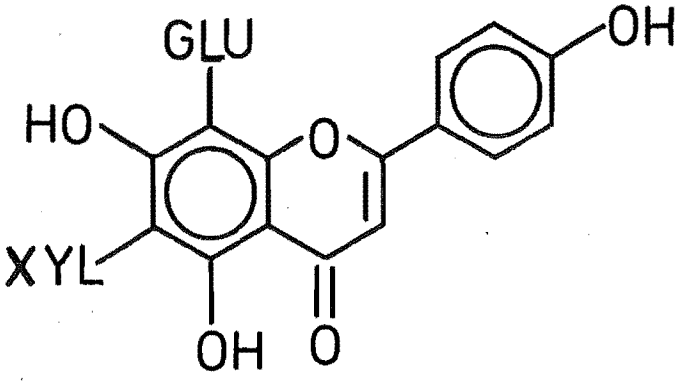
Ferulic Acid



o-AMINOPHENOL



Methoxyhydroquinone



VICENIN -1

Figure 1.1 Representative Phenolic Compounds of Wheat Grain

i) Vanillic Acid-a hydroxybenzoic acid
ii) Ferulic acid-a hydroxycinnamic acid
iii) 2-methoxyhydroquinone (aglycone)
iv) n-Alylresorcinol(generalised structure)
v) o-Aminophenol (aglycone)
vi) Vicenin 1-a C-Glycosylflavone.

peonodin glucosides and their acylated derivatives[37]. The presence of proanthocyanidins has been reported in the pericarp of immature wheat grain[38].

The bran of wheat and other cereals, notably rye, have been shown to contain significant quantities of 5-alkylresorcinol derivatives[39]. These may have significance as protective agents as they have allergenic and anti-nutritional properties.

Undoubtedly there are more phenolic compounds in the wheat grain that have not as yet been characterised. One group of biologically active phenols that are known to occur in cereal grains but about which little is known are the nitrogen-containing phenols. *O*-aminophenol has been detected in alkaline hydrolysates of wheat bran extracts[40] but nothing is as yet known of the form in which the aglycone occurs.

Studies of the physiological, nutritional and technological significance of cereal phenols are greatly hampered by the lack of information on their chemistry[19]. Cereal seeds pose particular problems for phytochemical studies owing to the relatively low concentrations of these compounds, their generally high degree of modification and conjugation, and the high levels of interfering material such as storage protein and carbohydrate.

1.3 Experimental Strategy and Outline

Three of the major questions that should be asked concerning phenolic compounds in cereal grains are :

1. How do they affect the quality of cereal grains and cereal products?
2. What phenolics are present in the mature grain?
3. How are they synthesized in the developing grain and how is this regulated?

The three sections into which this work has been divided each reflect an emphasis on one of these questions. In each section the studies have been guided by another overriding consideration:

"How do phenolics contribute to the colour of wheat and wheaten products?".

In the first section the relationship between the content of phenolics and phenol oxidase in flours and the colour of flour and bread has been examined. These parameters have been examined both for a series of flour streams from a commercial flour mill and for flours from several N.Z. wheat cultivars.

In the second section the proanthocyanidins present in wheat bran have been examined in order to determine the chemical nature of those which are present. These compounds are putative pigment precursors but have not been studied in wheat grain using chromatographic techniques, nor studied in the mature wheat grain.

The biosynthesis of phenolics in developing wheat grain has been examined in the final section. There do not appear to have been any previous studies of the

enzymes of phenolic biosynthesis or of the changes in phenolic composition in developing wheat grain. In addition, red- and white-grained wheat cultivars were compared in order to determine whether there was any evidence that they differed in their phenolic biosynthesis and metabolism during grain development.

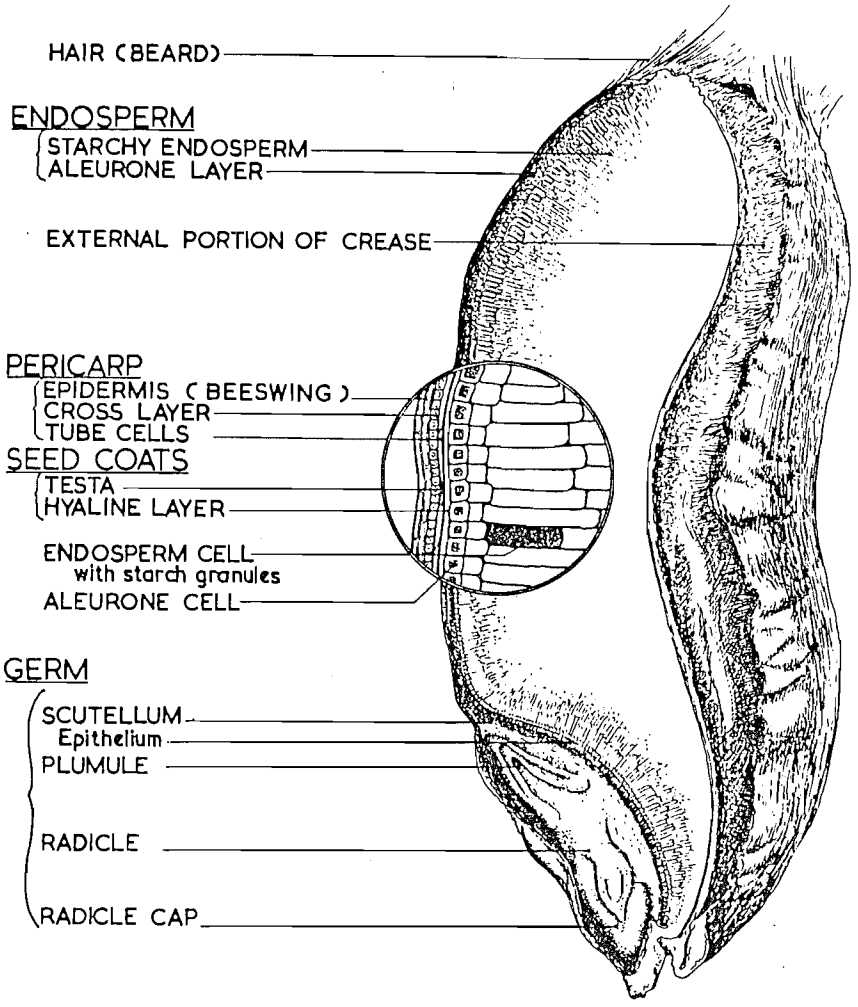


Figure 1.2 Longitudinal Section of a Wheat Grain (from Kent[158])

SECTION 2 *O*-DIPHENOL OXIDASE ACTIVITY, PHENOLIC CONTENT AND COLOUR OF NZ WHEATS, FLOURS AND MILLING STREAMS

2.1 Introduction

2.1.1 The Influence of Phenolics on Food Quality

The presence of phenolic compounds in foodstuffs can have a profound influence on their acceptability for processing or consumption[41]. Detrimental effects of phenols on food quality may commonly be a reflection of their role as protective agents.

The flavour, odour and palatability of many fruits and vegetables are markedly affected by their phenolic content[42]. Examples include the bitter flavonoids in citrus and astringent condensed tannins in fruits such as persimmon and quince. Simple volatile phenols produced during baking, curing,smoking and fermentation impart characteristic flavours to many processed or cooked products.

Tannins and other phenols may also reduce digestibility of plant material[9]. Tannins complex and precipitate digestive enzymes, whilst phenolic acids may limit the susceptibility of carbohydrate and protein to enzymic hydrolysis by substitution and crosslinking. Both effects are particularly important in determining forage crop quality for ruminant livestock[43,44].

There is evidence to suggest that some plant phenols in foods may possess toxic or nutritive properties[41]. Many phenolic compounds are weak, broad spectrum toxins and may have deleterious effects on humans when are consumed in large amounts ,such as condensed tannins in black tea or high-tannin sorghum varieties[45]. Some flavonoids have been shown to synergise the effects of Vitamin C and have themselves been claimed to constitute a "Vitamin P" activity[46].

As well as acting as antioxidants *in vivo* it is now known that phenolics, in particular the flavonoids, can play an important role in food processing and storage by retarding lipid-oxidative flavour changes[47].

Natural and modified phenolic pigments, notably the anthocyanins and other flavonoids, are responsible for the colouration of many plant foodstuffs. However phenolics are more important for their involvement in both the desirable and undesirable colour changes which occur during processing, cooking or spoilage of foods.

Browning Reactions in Foodstuffs

Discolouration or "browning" reactions have usually been classified and discussed in terms of "enzymic" or "non-enzymic" reactions. Such classification has probably tended to obscure the fact that a there is a wide variety of often inter-related reactions that could occur simultaneously (figure 2.1).

Non-enzymic browning is generally considered to be synonymous with the so-called "Maillard reactions". These are a sequence of reactions, initially involving the addition of a sugar carbonyl group to a primary amino group of an amino acid or protein, which ultimately gives rise to brown "melanoidin" pigments[48].

By contrast enzymic browning is defined as that initiated by enzymic oxidation of phenols[53]. The initial products of phenol oxidation are quinones, which readily undergo self-polymerization and attack by nucleophiles such as thiols or the ϵ-amino of lysine. Thio-ethers produced by such reactions are colourless whereas condensation of quinones with amino acids or proteins via amino groups leads to complex brown polymers[16].

Quinones exhibit dicarbonyl groupings similar to those which are the basis for the Maillard reaction and the Strecker degradation of amino acids. Therefore it may be generalised that most browning reactions in foods arise from carbonyl/amino group inter-reactions.

Phenolic compounds can also participate in a variety of non-enzymic browning reactions. This can occur through acid hydrolysis of glycosides or proanthocyanidins, spontaeneous or metal-catalysed air oxidation, colour changes due to pH or metal chelation, or condensation with aldehydes. It is likely that phenols can augment colour production in Maillard and caramelization reactions by condensation with reactive aldehyde intermediates[49].

The end products and stoichiometry of enzymic browning reactions are determined by the relative concentrations and structures of the phenols and amino acids or proteins present[50,16]. Whilst these reactions usually give rise to high molecular weight products of variable composition it now appears that under some conditions low MW coloured compounds may be formed, for example; the benzotropolones produced during the fermentation stage of tea manufacture[51].

Enzymes Involved in Food Browning Reactions

The enzyme activities responsible for oxidation and/or hydroxylation of plant phenols are peroxidase (POD, E.C.1.11.1.7), monophenol monooxygenase (E.C.1.14.18.1), *o*-diphenol oxidase (*o*-DPO, E.C. 1.10.3.2) and *p*-diphenol oxidase (or laccase, E.C.

1.10.3.1)[12]. Phenolase enzymes from plants typically exhibit both monophenol monooxygenase and o-diphenol oxidase activities, though the observed ratio of activities may vary widely according to the particular enzyme source[52]. Enzymic browning reactions are generally considered to result from the oxidation of *o*-diphenols by phenolases[53], though oxidation by peroxidases may be important in some situations.

Phenolases are Cu-containing enzymes and typically show broad substrate specificity for both hydroxylase and oxidase activities[52]. They may be soluble or membrane-bound and have been recently shown to occur exclusively in plastids in intact plant tissues[153]. Association of oxygenases with membranes may be particularly important for the compartmentalization and regulation of the synthesis of their relatively polar and biologically active products[12].They are subject to modification of activity by a variety of treatments including detergents, proteolysis,and a variety of organic and inorganic compounds. They are usally subject to inhibition by their own products as well as by some phenols. The commonest natural substrates for *o*-DPO are hydroxycinnamic acid esters such as chlorogenic acid (fig. 4.11) or flavan-3-ols such as (+)catechin (fig. 3.1).

2.1.2 Phenolics and Phenol Oxidases in Wheat Grain: Their Role in Colouration and Discolouration of Wheaten Products

Colour is an important quality factor in determining consumer appeal of wheaten products[54]. White wheats that produce lighter-coloured products generally command a better price on the open market than red wheats. To date the NZ wheat crop has largely consisted of red wheats which possess resistance to pre-harvest sprouting. In modern roller milling there is strong pressure to utilize as much of the grain as possible for high value end-uses. This has meant that there has been a trend towards higher extraction rates and utilization of milling by-products in baked goods, with an associated increase in the problems of maintaining colour quality.

The measurement of flour and product colour poses many difficulties and until recently the miller's and baker's trained eye were the most practical means of assessment. The Pekar (slick) test has been the usual method for the subjective assessment of flour colour. Flour pigment content has been determined by measuring absorbance of water-saturated *n*-butanol or other solvent extracts. Purpose-built reflectometers have been used for measurement of flour colour for some years, particularly the Kent-Jones and Martin colour grader[55]. This device has also been used for the determination of bread crumb colour[57] though it does not appear to have been widely used for this purpose. In recent years the development of more flexible microprocesser-controlled

diffuse reflectance spectrophotometers has provided the means for more quantitative and objective comparisons of flour, meal and cereal product colour[56]. Few studies of the chemistry of cereal product colour and discolouration to date have employed objective quantitative measures of colour.

Problems of undesirable colour in cereal products occur from time to time in various countries, especially with the introduction of new cultivars or processing methods. One area where colour is particularly important for determining consumer appeal is in the manufacture of pasta and noodles. Durum wheat semolinas with maximum yellow pigmentation but minimum brown pigmentation are preferred for pasta manufacture. Development of grey or brown colours and loss of yellow colour are undesirable during manufacture. A copper-protein complex has been associated with brownness in macaroni produced from certain durum wheats[58]. Production of Asian-style noodles from bread wheat flour involves treatment with alkali and the high pH imparts a characteristic yellow colour to the noodles. Discolouration can sometimes result, particularly if bran or germ contamination is present[59].

Undesirable browning of wheaten products has been encountered with certain wheats grown in southern France. Studies there suggested that there was a strong correlation between flour-paste browning, as determined by reflectance measurements, and activities of POD and *o*-DPO in the flour[60].

Cake and bisuit manufacture require flours of minimum yellowness and maximum whiteness, and here the colour is most likely determined by Maillard-type reactions[63].

In India dwarf wheats fom the CIMMYT* breeding programme in Mexico have proved to be unsatisfactory for making chapatis due to excessive darkening of doughs prepared from these varieties[61]. Studies there suggested that this was caused by enzymic browning due to higher levels of phenolics and phenol oxidases in these wheats compared to traditional varieties[62].

Although demand for white bread has declined somewhat in recent years the control of colour in bread and otherwheaten products is still a matter of importance to bakers. Following the raising of flour extraction rates after World War II NZ mills have, on occasions, produced flours giving bread with a slightly grey crumb colour. Incidence of "dark crumb" as it is known varies between mills, seasons, cultivars and growing areas, whilst milling experience indicates it is more common early in the season and when grists are milled at too low a moisture content[64]. Dark crumb appears to be less

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* International Centre for Maize and Wheat Improvement

frequent in breadmaking using mechanical dough development (MDD) than in slower traditional bulk fermentation(R.W.Cawley pers.comm.).

Early work in NZ at the Wheat Research Institute (W.R.I.) by Bird and Stern[65] on darkening of germ doughs established that this darkening was due to enzymic oxidations and that fermentation provides favourable conditions for these. Later work showed that bran and germ in combination were most effective at producing dark crumb in flours, suggesting that darkening involves an interaction of components from each (R.W.Cawley pers.comm.).

A number of substances have been suggested to contribute to the colour of flour and wheaten products, though in many cases there is still a lack of understanding of the chemistry of the individual compounds involved. Carotenoids, principally xanthophyll, impart a yellow colour to flour and to pasta products. C-glycosyl-flavones also contribute some yellowness to flours and have been suggested to contribute to the undesirable darkening of pasta by undergoing oxidation[54]. The yellow-green colour of Asian-type noodles prepared with alkali is probably due to the indicator properties of these compounds[85].

The red-brown pigments of wheat bran have been considered to be phlobaphenes i.e. products of polyphenol oxidation[49]. In general little is known of the chemistry of these and other bran pigments.

There is considerable evidence to suggest that the methoxyhydroquinone glucosides, which are predominantly located in the germ , may contribute to wheat grain browning reactions. Early studies[31,66] showed that 2-methoxy-benzoquinone and 2,6-dimethoxybenzoquinone are produced from these compounds in fermented doughs, presumably due to the action of β-glucosidase and enzymic or air oxidation. Cawley examined the action of the hydroquinone derivatives present in germ as dough improvers[67] and their role in dough discolouration. These compounds, alone or in combination with amino acids, were shown to give rise to a variety of colours in breadmaking. Later work showed addition of caffeic acid could similarly affect crumb colour (R.W.Cawley pers.comm.).

Wheat grain has been shown to contain a variety of oxidative enzymes[68] and appears to contain higher levels of several of these compared to other cereals[70,71,72]. The "phenol test", in which grains are incubated in solution containing phenol until staining of the seedcoat occurs is commonly used by cereal breeders to identify cultivars and indicates great intervarietal variability in the distribution and amount of oxidative activity[69].

The presence of phenolase activity in bran was first reported by Bertrand and Muttermilch in 1907[73] and subsequently there have been numerous studies of phenol oxidase activities in developing, mature and germinating grain. *O*-DPO activity in the mature grain is localised mainly in the bran and only slight activity is normally present in flour and germ[74]. Electrophoresis indicates that up to ten *o*-DPO isoenzymes may be present[68]. *O*-DPO isoenzymes from durum and bread wheats have been purified and extensively characterised by Interesse and co-workers who showed that these possessed only very low activity towards monophenols[75,76]. However studies of phenolase activities in developing grain have shown that monophenol oxidase activity can be detected during grain maturation[77]. Levels of mono- and diphenol oxidase activity increase significantly on germination and it has been suggested that this may explain problems of discolouration experienced with sprouted wheats[77].

Developing and mature wheat grain contain a number of peroxidase isoenzymes in the seedcoat, endosperm and embryo[68,78] .Several peroxidases have been purified and characterized from wheat germ[79]. A study of eleven German and Canadian wheats revealed no correlation between their peroxidase activity and baking performance[80].

The presence of high *o*-phenylenediamine oxidase activity has been reported in mature and germinating wheat grain[70]. The significance and nature of the enzymes involved is unknown, though oxidation of aminophenolic substrates could conceivably give rise to coloured products in grain development or in browning reactions[19].

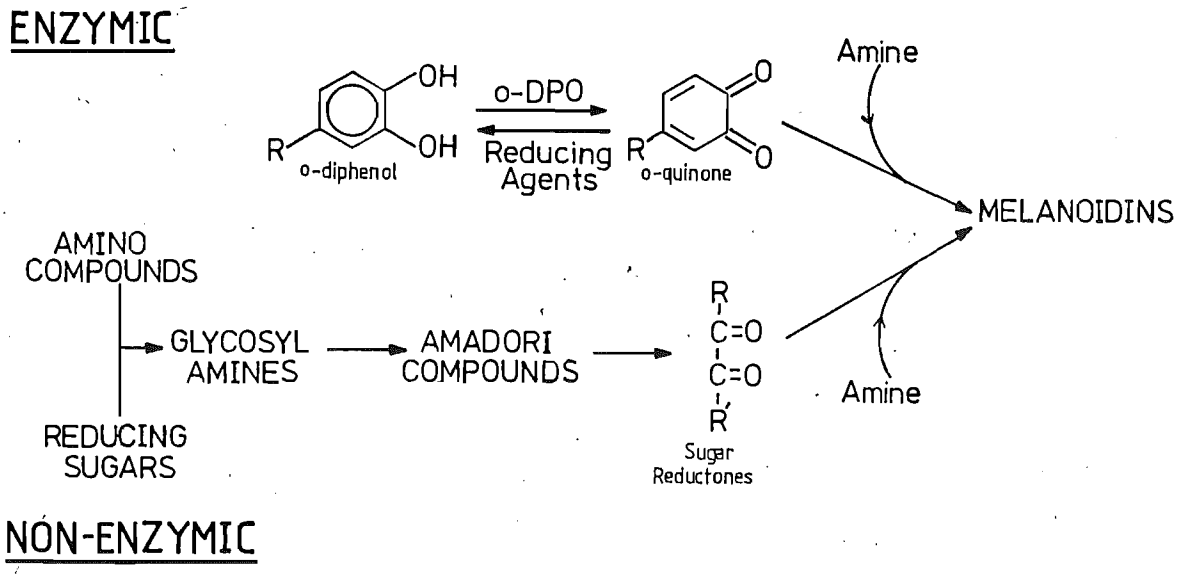


Figure 2.1 Generalised Mechanisms of Food Browning Reactions

2.2 Phenolics, *O*-Diphenol Oxidase, Flour Colour and Bread Colour of Flour Milling Streams

2.2.1 Materials and Methods

Materials

4-Methyl-catechol and 2-methoxyhydroquinone(MHQ, 1,4-dihydroxy-2-methoxybenzene) were obtained from Fluka AG (Switzerland); 4-methyl-catechol was recrystallised from hexane before use. Vanillin was obtained from B.D.H. Ltd.. Ferulic acid, gallic acid and (+)catechin were obtained from Sigma Chemical Co.(U.S.A.). Gallic acid and (+)catechin were recrystallised from hot water for use as analytical standards. Schaftoside (6-C-glucosyl-8-C-arabinosylapigenin) was a kind gift from Dr. K.R.Markham, Chemistry Division, DSIR, Petone. All other reagents were obtained from Sigma or B.D.H. and were AR grade. All solvents were distilled in an all-glass apparatus before use.

Folin-Denis reagent was prepared according to A.O.A.C. official methods[81].

Amberlite XAD-2 resin was obtained from Sigma and was exhaustively extracted with acetone in a soxhlet apparatus before use[143].

Samples of 16 individual milling flour streams were obtained from D.H.Brown Ltd.Flour Millers,Christchurch. These were prepared from the milling of a mixed grist consisting of approximately 70% cv Rongotea and 30% cv Oroua at an extraction rate of 78%. (The principles of flour milling are illustrated in figure 2.5). Wholemeal samples were prepared using a Udy Cyclone mill. All flour and meal samples were sealed in plastic bags and stored at -20°C until analysis or baking.

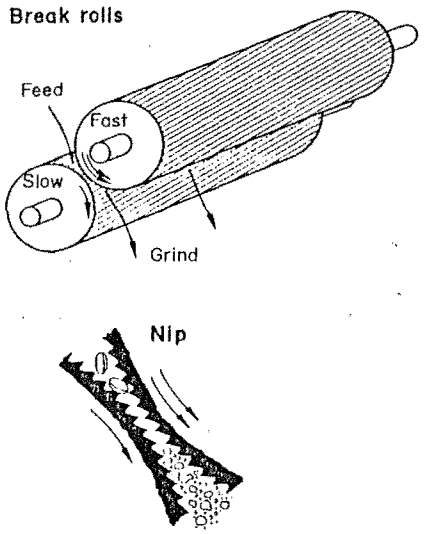
Methods

Test baking was performed on the 125g MDD testbake facility at Wheat Research Institute (DSIR), Christchurch.

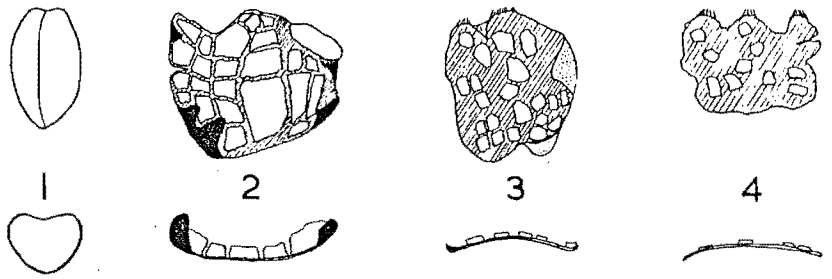
Flour colour was measured using a Kent-Jones and Martin Series 3 Colour grader[55]. Bread crumb colour was also measured in the colour grader by the method of Pomeranz[57] but without addition of starch. Crumb colour was also ranked subjectively.

Extraction and Analysis of Soluble Phenolics

10g samples (fresh weight) of flour or meal were extracted by shaking for 2hrs in 200ml 75% aqueous acetone in 250ml centrifuge bottles flushed with nitrogen. These



Pair of break rolls showing flutes, with enlarged view of the "nip"



Stages in opening out of the wheat grain and scraping of endosperm from bran by fluted rolls of the Break system. 1, Whole wheat grain; 2, I Bk. tails; 3, II Bk. tails; 4, III Bk. tails. *Upper row:* plan view (looking down onto inside of the bran in 2–4). *Lower row:* side view. In 2–4, endosperm particles adherent to bran are uncoloured; inner surface of bran, free of endosperm, is hatched; outer side of bran curling over is shown in solid black; beeswing, from which bran has broken away, is shown dotted.

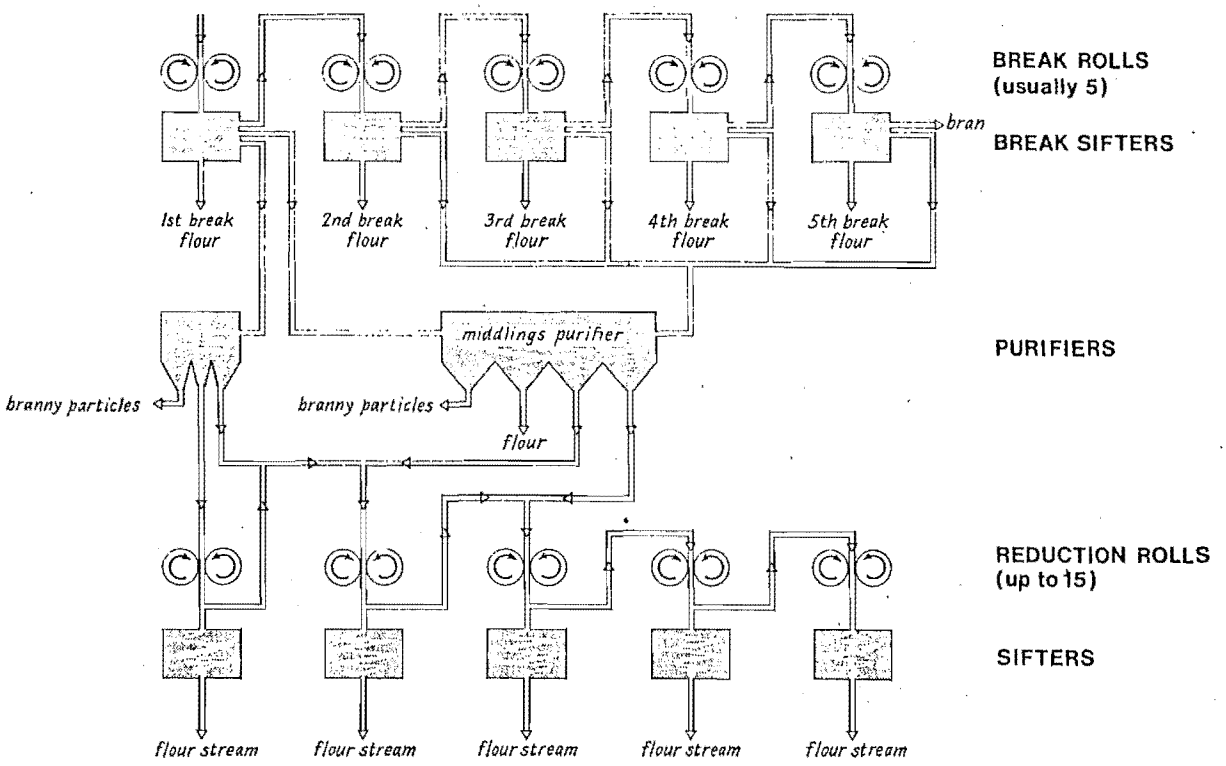


Figure 2.2 Principles of Flour Milling (from Kent[158]) and Generalised Scheme of a Flour Mill (from Fox and Cameron[224]).

were then centrifuged at 5000G for 15 min.,the supernatant decanted and reduced to 50 ml at 25°C *in vacuo*. Aliquots of this extract were analysed for total phenols by the Folin-Denis assay, and for flavanols by the vanillin-sulphuric acid assay[83], using gallic acid and (+)catechin standards respectively. Duplicate extracts were prepared and duplicate assays were performed on these.

PC Inspection of Folin-Reactive Phenols

A 30g sample of cv Oroua grain was ground on a Casella grain mill to pass a 1.5 mm screen. The meal was stirred in 400ml 75% aq. acetone with N_2 purging for 30 min and the extract was filtered through Whatman #1 filter paper. The acetone was removed by rotary evaporation at 25°C and the aqueous residue was extracted with three successive 50ml portions of hexane. The extract (pH approx. 5) was then applied to a 2.5cmx20cm column of Amberlite XAD-2 resin equilibrated with distilled water and washed with a further 100ml distilled water. The column was eluted with 200ml methanol and the methanolic eluate was taken to dryness at 25°C *in vacuo*.

The methanolic eluate was redissolved in 3ml of 80% aq.methanol and 20μl aliquots were applied to 20cm x 20cm sheets of Whatman #1 chromatography paper for ascending PC in BAW (Butanol/acetic acid/water 40/10/22) followed by 5% acetic acid in the second dimension[84]. Developed sheets were examined in UV and daylight before and after fuming with ammonia, after spraying with Folin-Denis reagent followed by saturated sodium carbonate or after spraying with 10% aq. *p*-toluenesulphonic acid followed by heating at 110°C for 10 minutes.

The major Folin-reactive phenolic was further purified by 1D-PC on Whatman 3MM paper developed with BAW from extracts prepared as above. The phenolic was located on strips cut from the chromatogram with *p*-toluenesulphonic acid. The bands corresponding to the phenolic were then extracted with 50% aq. methanol and the extracts were purified further by gel filtration on Sephadex G-25 eluted with water. Fractions were examined by TLC on Schleicher & Schull Avicel cellulose sheets developed with BAW and the fractions containing the phenolic were freeze-dried.

UV spectra were recorded on a Pye Unicam SP1800 double beam spectrophotometer in methanol before and after addition of either; sodium methoxide, aluminium chloride or sodium tetraborate.

^{13}C- and ^{1}H NMR spectra were recorded in d_6-DMSO with dioxane as an internal standard.

Positive ion FAB mass spectroscopy was performed at Biotech.Div. DSIR. The phenolic was dissolved in glycerol prior to insertion on the probe tip.

Detection of Browning Substrates

Crude enzyme extracts were prepared by grinding 15g cv Oroua wholemeal with acid-washed sand in 50ml chilled 0.05M phosphate buffer, pH 6.6, in a mortar and pestle. The resulting slurry was filtered through two layers of Miracloth and centrifuged for 15min at 20,000G at4°C. The supernatant was used as a crude enzyme source.

In attempts to detect natural substrates for enzymic browning[42] 1D and 2D paper chromatograms of cv Oroua phenolics,prepared as above, were dipped in 2.5% aq. arginine hydrochloride and then either in the crude enzyme preparation or extraction buffer. Pieces of Whatman 3MM paper spotted with 10, 100 and 500 μg of 4-methyl-catechol, caffeic acid, chlorogenic acid (+)catechin or L-DOPA were treated similarly. Treated chromatograms were held in a closed container in a water-saturated atmosphere, in the dark, at room temperature and observed over 48 hours.

Assay of o-Diphenol Oxidase Activity

Standard Assay Conditions

Assays were performed at 30°C using a Clark oxygen electrode (Yellow Springs Instrument Co.,U.S.A.) fitted with a polyethylene membrane. The electrode was calibrated with air-saturated water. 0.2g samples of flour were incubated with stirring in 3ml air-saturated 0.05M citrate-phosphate buffer pH5.6 for 10 minutes to allow establishment of a stable base rate of oxygen consumption; then 0.1ml of 0.31M 4-methyl catechol was then injected to give a final concentration of 10mM. *O*-diphenol oxidase activity was determined from the difference between the basal rate of oxygen consumption and the initial rate following addition of substrate. Relative rates of *o*-DPO activity were expressed as mV.min-1.

Determination of pH Optimum for o-Diphenol Oxidase

A sample of straight-run flour, also obtained from D.H.Brown Ltd mill, was assayed as above in 0.05M citrate-phosphate buffers from of pH5 to pH6. Assays were performed in triplicate.

Determination Of Germ Index

Germ index, a measure of relative germ content as soluble C-glycosyl-flavones, was determined by the method of Stenvert and Murray[85]. Duplicate 5g flour samples were homogenized for 5 minutes in 50ml distilled water using an MSE homogenizer.

Extracts were then centrifuged for 5min at 5000 rpm and the supernatant was clarified further by filtration through a Whatman GF/C filter.

A 5ml aliquot of the extract was basified by addition of 50 μl of 50% NaOH and the absorbance was measured in 1cm glass cells at 385nm.

Statistical Analysis

Data analysis was performed using SAS routines. Raw data variables were examined for normality using PROC UNIVARIATE, correlation coefficients were obtained with PROC CORR ,regression coefficients with PROC REG and ANOVAs with PROC GLM[92,93]. Correlation coefficients were compared by transformation to normal variates

$$z_i = \tfrac{1}{2}\log_e(1+r/1-r)$$

and the significance of the difference between these

$$D = z_1 - z_2 / \Sigma(1/n_i - 3)$$

was tested against the normal distribution. A significance level of 5% (p=0.05) was used for this and all other statistical tests.

Slopes of regression lines were compared by calculating

$$T = b_1 - b_2 / \sqrt{(SE_{b1} + SE_{b2})}$$

and comparing with $t_{n1+n2-2\ df}$

All values for parameters estimated in experiments are reported in tables and graphs as the mean and standard error of the mean (SEM).

2.2.2 Results and Discussion

O-Diphenol Oxidase in Wheat Flour

The pH optimum of wheat flour *o*-diphenol oxidase was determined over the range pH5-pH6, which is the typical pH range of a fermenting dough, and this indicated that activity was optimal around pH 5.6 (Fig. 2.3). Interesse *et al*[87] have shown that purified wheat grain *o*-diphenol oxidase has two optima at 6.9 and 5.3. All subsequent assays were performed at pH 5.6 because it was considered to be more relevant to dough browning reactions and non-enzymic oxidation of 4-methyl catechol was negligible at this pH. Other workers who have used the oxygen electrode for assay of wheat *o*-diphenol oxidase have generally assayed at higher pH and utilized catechol as substrate[74,88]. 4-Methyl-catechol has generally been found to be the best substrate for wheat[87] and other *o*-diphenol oxidases.

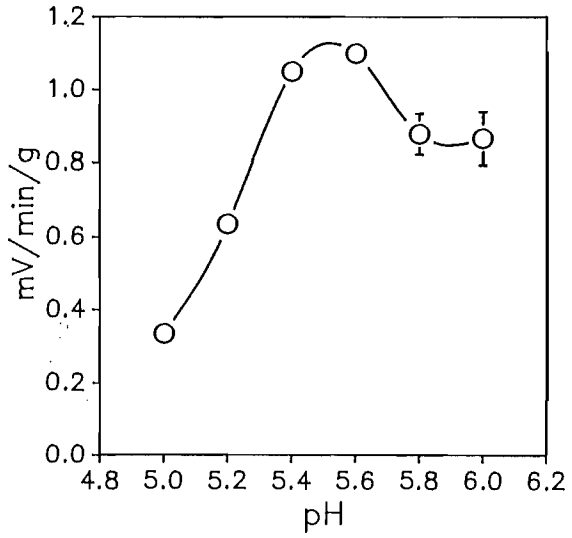


Figure 2.3 pH Optimum of Wheat Flour o-Diphenol Oxidase. (Points represent mean ± std. error for n=3 assays.).

The oxygen electrode provides a rapid, simple means for determination of relative *o*-diphenol oxidase activities in flour samples by avoiding the difficulties and uncertainties associated with extraction[88]. The enzyme can generally only be partially solubilised and has been shown to be associated with a variety of organelles.[52]

The mill flour streams showed a wide range of *o*-diphenol oxidase activity ranging from 0.007 to 7.44 mV/min/g. The straight-run flour showed an activity of 1.10 mV/min/g.

Soluble Phenolics in Wheat Flour

The phenolic content of all milling flour streams, as determined by three simple quantitative assays, varied over a range of approximately one order of magnitude. Relative soluble C-glycosyl-flavone content (germ index) in the mill flour streams ranged from 0.09 to 1.1.AU_{385nm}. Total soluble phenols (Folin-Denis phenol assay) showed a range of 0.20-1.61 mg/g dry wt and flavanols (vanillin-sulphuric acid assay) 22.6-141.7 μg/g dry wt.

The Folin-Denis assay relies on the oxidation of phenols under alkaline conditions and thus is prone to interference by reducing agents[89]. The vanillin-sulphuric acid assay is also prone to interference by reducing agents due to the oxidizing properties of sulphuric acid [90] and possibly to aromatic amines[177]. The related Folin-Ciocalteau and Vanillin-HCl assays appear to be less susceptible to such interference and may be more satisfactory for assay of crude extracts.

The phenols reacting with Folin-Denis reagent were examined further by spraying 2D paper chromatograms of wheat grain extracts with the reagent. A number of compounds reacted to give blue colour with the reagent (Fig.2.4, Table 2.1).

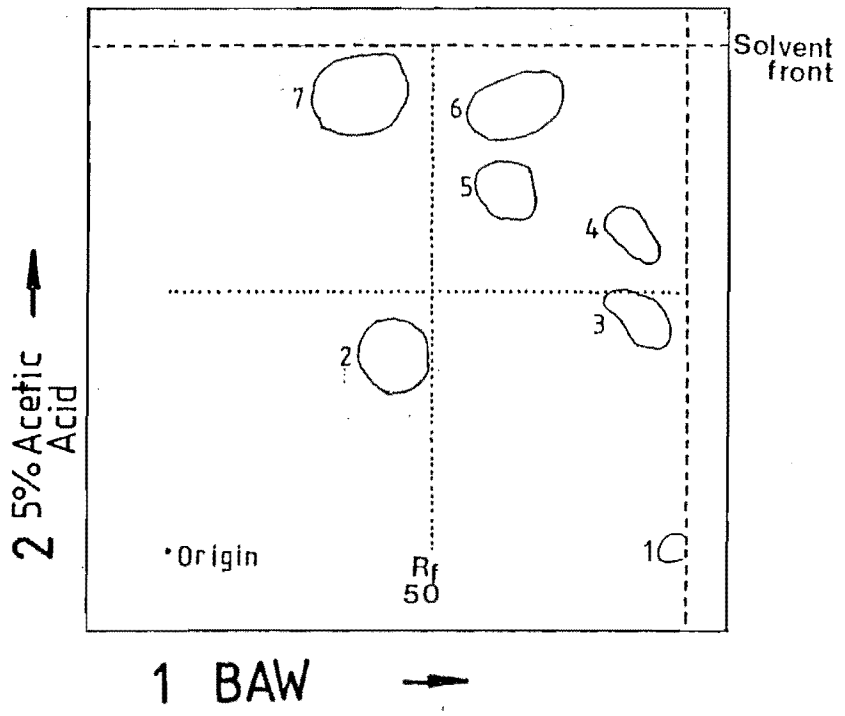


Figure 2.4 Paper Chromatography of Folin-Reactive Phenolics from Wheat Grain cv Oroua

Table 2.1 Paper Chromatographic Characteristics of Wheat Grain Phenolics

Spot Number	Rf BAW	Rf 5% HAc	Colour in UV	Colour in UV+NH3	Probable Identity
1	100	0	Blue	Blue	Unknown
2	50	42	Purple	Green	Schaftoside (and other flavones)
3	94	50	Blue	Light Blue	Ferulic Acid
4	94	61	Blue	Light Blue	Ferulic Acid
5	67	74	Blue	Light Blue	Ferulic Acid
6	72	95	Blue	Light Blue	Sterol Esters?
7	33	98	Faint Dark	Faint Dark	MHQ-tri-Glucoside

Ferulic and vanillic acids and schaftoside were tentatively identified by their colour under UV and visible light after treatment with ammonia and by their chromatographic mobility relative to authentic standards. Descending paper chromatography on larger sheets showed that the zone corresponding to schaftoside could be resolved into several spots with fluorescence characteristics typical of C-glycosyl-flavones. The presence in wheat germ of schaftoside and related isomers has been reported by Wagner *et al*[28]. Ferulic acid is a major phenolic acid in wheat grain but is generally only present in trace amounts as the free acid[26]. The unidentified spots with UV fluorescence characteristic of hydroxycinnamic acids are possibly sterol or stanol esters of ferulic acid which have recently been reported to be major soluble forms of this acid[25].

The spots reacting most strongly with Folin reagent were those containing schaftoside (and presumably other flavones) and an unidentified spot with low mobility in BAW but very high mobility in 5% Acetic acid. As this spot appeared to be quantitatively the major phenol reacting with the reagent its nature was examined further following purification by preparative 1D-PC and gel filtration.

Acid hydrolysis of the purified phenolic compound yielded an aglycone which co-migrated on cellulose TLC in BAW (R_f=95) with authentic 2-methoxyhydroquinone (MHQ) and similarly autoxidized on the TLC sheet to become visible as a dark spot. The nature of the aglycone was confirmed by UV spectroscopy (UV_{max} in MeOH 291nm,UV_{max} in MeOH+NaOMe 272nm decl.).^{1}H-NMR of the glycoside also confirmed the presence of the MHQ nucleus and showed signals characteristic of of $\beta(1\rightarrow4)$ bonds between glucose molecules (Doublets at 4.33 and 4.39 ppm). FAB mass spectroscopy in glycerol indicated a molecular weight of 626 (627 $M+H^+$,719 M+Glycerol), suggesting that the phenolic was a triglucoside of MHQ (fig. 2.5).

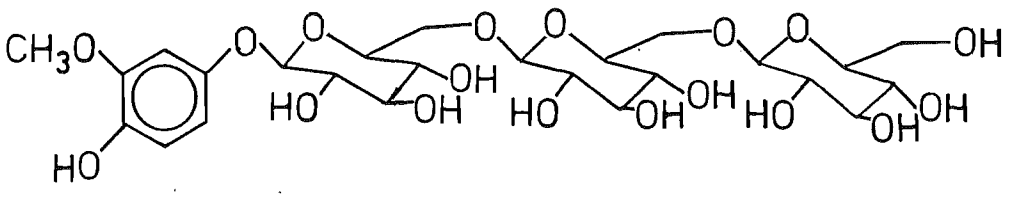


Figure 2.5 MHQ Triglucoside {(3-methoxy-4-hydroxyphenyl)-ß-cellotrioside}

Comparison of Milling Streams

The Kent-Jones colour grader uses a filter with maximum transmittance at 530nm and gives a reading that reflects primarily the amount of bran present in flour[55], although reflectance of endosperm components may also be significant at lower colour grades. Flour colour grade was highly correlated with crumb colour grades for this series of flours (table 2.2, figure 2.6), in agreement with previous studies of mill flour streams at W.R.I.(Marcella Ross pers.comm.) and the observations of Pomeranz[57]. Bread giving a crumb colour grade greater than 10.5 Kent-Jones units by this method was generally judged as dark crumb. Darker flours gave flour and/or crumb colours with off-scale grader readings and were excluded from subsequent analysis. The narrow working range of the Kent-Jones colour grader prevents its' use in studies of wholemeal bread or paste colour.

In studies of this kind it is necessary to establish whether objectively measured colour relates to visual appearance. The perception of bread crumb colour is greatly affected by crumb texture[91] but determination of crumb colour grade in a slurry removes the influence of this effect. Flour and crumb colour grade were also correlated with visually assessed crumb colour rank. This correlation confirms that these objective measures of colour relate closely to subjectively perceived colour and thus may be considered relevant to consumer appeal and preference.

All other raw data variables measured for the flour samples were highly correlated with flour colour grade, crumb colour grade and crumb colour rank (table 2.2, figs. 2.2-2.9). Since crumb colour was the response variable of interest all predictor variables have been graphed against it, though it should be noted that all of these showed higher correlations with flour colour. The plots of several of these variables suggested a log-linear relationship with colour grades so the correlation coefficients obtained from the raw data were compared with those obtained from correlation analysis of colour grades and $\log_e$-tranformed data. These comparisons indicated that only for the germ index was the correlation coefficient significantly improved at the significance level $p=0.05$, which suggested that there was a non-linear relationship between this variable and colour grades.

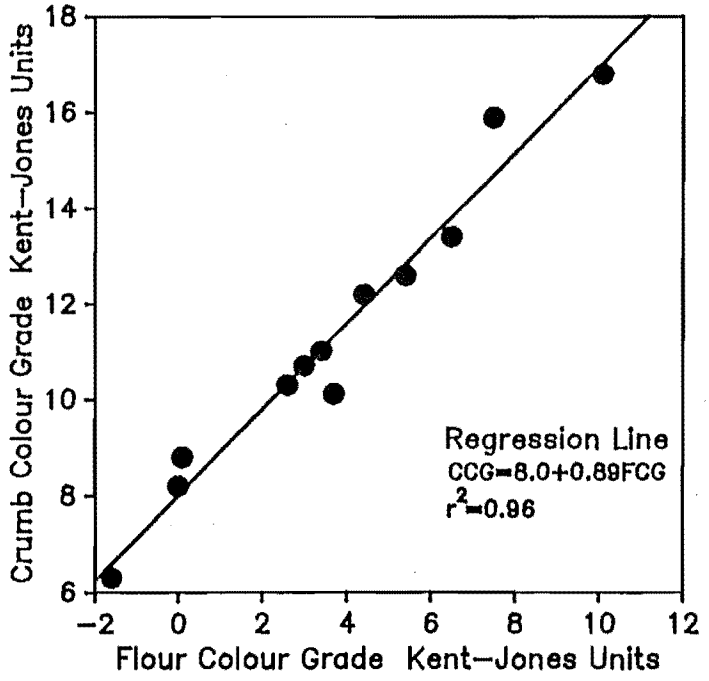


Figure 2.6 Flour and Bread Crumb Colour of Milling Flour Streams.

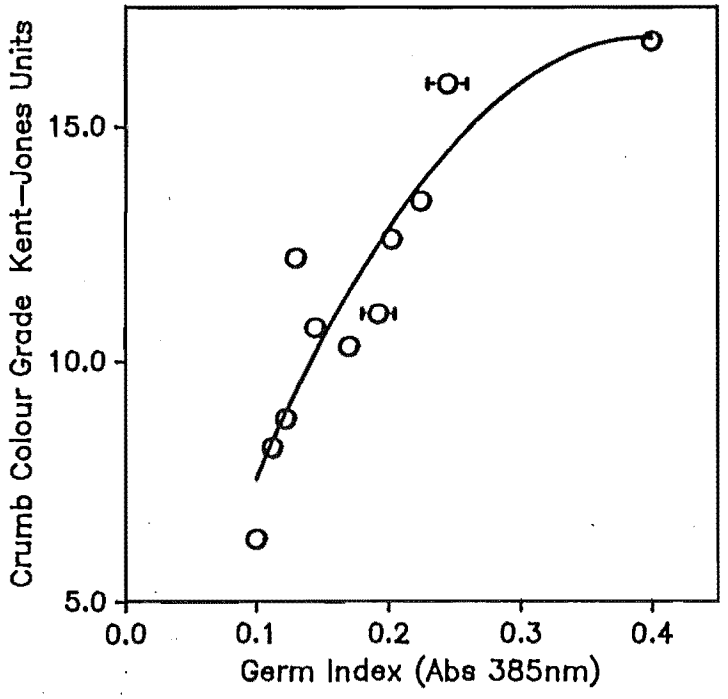


Figure 2.7 Germ Index and Bread Crumb Colour of Milling Flour Streams. (Points represent mean ± std. error for n = 2 assays)

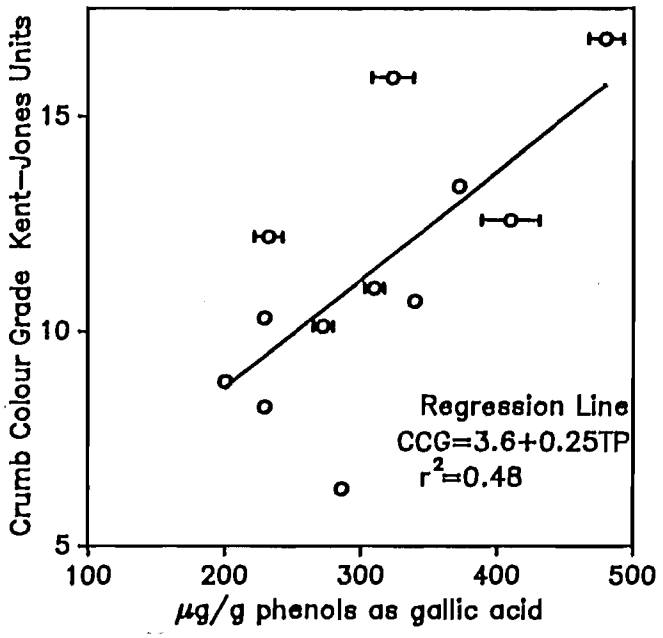


Figure 2.8 Total Phenolics and Bread Crumb Colour of Milling Flour Streams. (Points represent mean ± std. error for n=2 extracts and n=2 assays.)

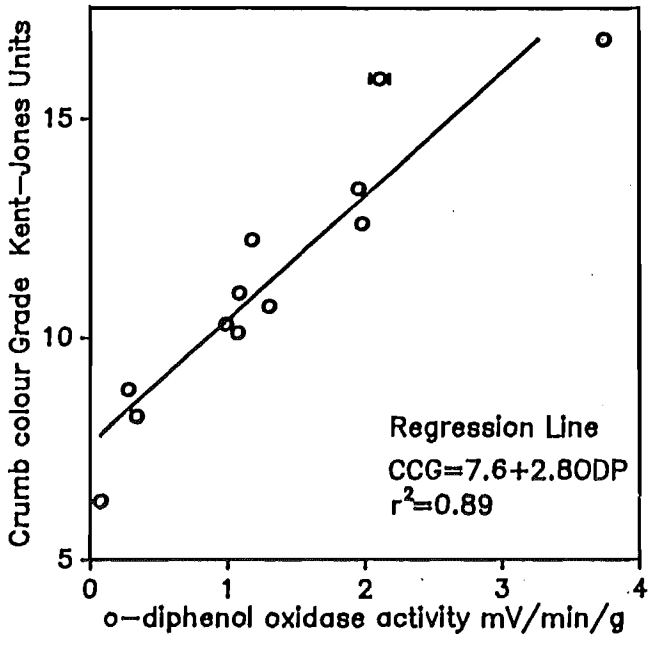


Figure 2.9 *O*-Diphenol Oxidase Activity and Bread Crumb Colour of Milling Flour Streams. (Points represent mean ± std. error for n=3 assays.)

Table 2.2 Results of Correlation Analysis of Mill Stream Data (Values are Pearson's or Spearmans' Correlation Coefficient Followed by n and significance levels)

Variable	Flour Colour Grade (Pearsons' Correlation Coefficient)	Crumb Colour Grade† (Pearsons' Correlation Coefficient)	Crumb Colour Rank (Spearmans'Rank Correlation Coefficient)
Flour Colour Grade	-	-	0.996 (11,0.0001)
Crumb Colour Grade	0.981 (15,0.0001)		0.987 (11,0.0001)
Germ Index	0.954 (28,0.0001)	0.867 (24,0.0001)	0.970 (28,0.0001)
Log_e Germ Index	0.973* (28,0.0001)	0.908* (24,0.0001)	-
O-Diphenol Oxidase Activity	0.977 (30,0.0001)	0.935 (22,0.0001)	0.967 (30,0.0001)
Soluble Total Phenols	0.893 (60,0.0001)	0.679 (48,0.0001)	0.866 (60,0.0001)
Soluble Flavanols	0.697 (60,0.0001)	0.328 (48,0.02)	0.625 (60,0.0001)

* Denotes correlation coefficient significantly increased ($p<0.05$) following Log_e transformation of germ index.

† Crumb Colour of Loaves from MDD testbake.

The inter-relationship of flour colour, crumb colour and *o*-diphenol oxidase activity observed here has been compared with that observed in a series of flours prepared from different cultivars in section 2.4.2 .

Within the series of break and reduction rolls there was a general trend towards higher values for all parameters with successive rolls (table 2.3). This trend has been noted for germ index by Stenvert and Murray[85].

The non-linear relationship observed between germ index and colour grade suggests that germ contamination of flours may increase more markedly than does bran fragmentation later in the series of break or reduction rolls when roller settings are

closer. Therefore at higher extraction rates a relatively small increase in extraction rate or subtle change in milling conditions may result in a relatively large increase in germ contamination and potential for discolouration.

Table 2.3 Spearman Rank Correlation Coefficients from Rank Correlation Analysis of Flour Stream Variables(Flour Colour etc.) and Roller Number. (* designates correlation coefficient significant at p=0.05).

Variable	Break Series(n=4)	Reduction Series(n=9)
Flour Colour	0.95*	0.80
Crumb Colour	0.98*	0.80
Germ Index	0.95*	1.00*
o-DPO Activity	0.98*	1.00*
Total Phenols	0.87*	1.00*
Flavanols	0.68*	0.80

O-diphenol oxidase is known to be localized in the bran[74] and the high correlation observed here confirms the value of flour colour grade as a measure of bran contamination. Flavanols are also present in the bran but it is likely that the values obtained here largely reflect interference by reducing material and other phenolic compounds[177] (See Sec 3.3.3).

Paper chromatography suggested that the predominant phenols reacting with Folin-Denis reagent were C-glycosyl-flavones and MHQ-triglucoside. Soluble C-glycosyl-flavones are present exclusively in the germ[26] but histochemical studies[30] suggest that MHQ derivatives may be present in the seedcoat tissues which are present in bran. This could suggest that the Folin-Denis assay is measuring components from **both** bran **and** germ. Different flour streams may also have marked qualitative differences in their phenolic content.

The trends evident from this data may be analogous to those observed in the concentration of minerals and vitamins in flours, where the nature of the relationship

between concentration and flour extraction rate depends on the particular distribution of the components within the grain[86] (fig. 2.10). The differences in the shapes of the relationships observed when *o*-DPO activity, germ index and total phenols are plotted against flour or crumb colour grades could reflect the fact that these parameters measure components localised in bran, germ and bran plus germ respectively.

Detection of Browning Substrates

Attempts to use enzyme preparations to detect substrates for enzymic browning on chromatograms of wheat phenolics did not reveal any marked browning. Stored chromatograms developed three brown zones which appeared to correspond to the major C-glycosyl-flavones and to MHQ-triglucoside noted in chromatograms sprayed with Folin reagent. The failure to observe any reaction may be due to some of the enzymes, substrates or other compounds involved remaining bound to insoluble material. This would seem to be the case since the surface of pellets remaining after decantation of the supernatant in enzyme extracts turned a dark red/grey colour if left overnight. Bird and Stern[65] noted that browning of wheat extracts was negligible in the absence of solid bran particles.

When phenolic standards were sprayed with crude enzyme preparations (+)catechin rapidly yielded an intense red/brown colour in the presence of arginine even at the lowest loading used (100μg). 4-Methyl-catechol also yielded a weaker red colour, whilst caffeic and chlorogenic acids gave weak green colours. Control sheets without enzyme showed no visible colours after 48 hours. (+)Catechin has been shown to be a good substrate for wheat *o*-diphenol oxidase[87] and red bran pigments are usually considered to be the products of oxidation of flavanols such as catechin by *o*-diphenol oxidase[38]. These observations suggest that oxidation of relatively small amounts of these compounds in the presence of protein or amino acids could give rise to such red pigmentation.

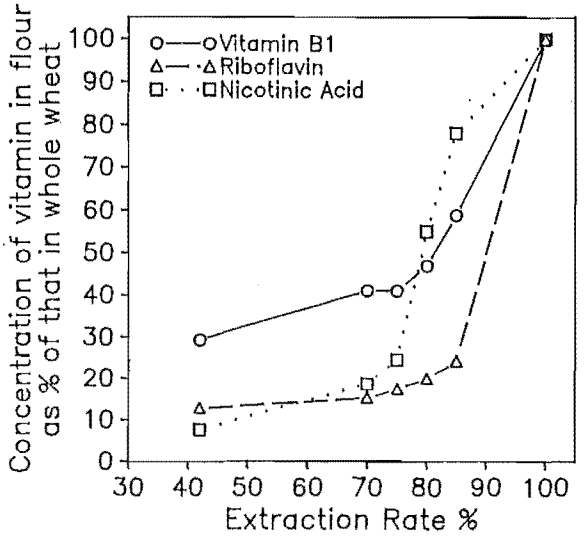


Figure 2.10 Nutrient Composition of Flours of Various Extraction Rates in Relation to That of Whole Wheat (from McCance *et al*[86])

2.3 Phenolics and *O*-Diphenol Oxidase Activity in Grain of Six NZ Wheat Cultivars

2.3.1 Materials and Methods

Samples of pure seed of six bread wheat cultivars (1986 harvest) were obtained from Crop Research Division,D.S.I.R. (Lincoln), and ground in a Udy Cyclone mill for subsequent analysis. The wholemeals were stored in sealed plastic bags at -20°C until analysis.

Analysis of soluble phenolics and *o*-DPO activity, and subsequent statistical analysis were performed as described in section 2.2.1

2.3.2 Results and Discussion

Comparison of this set of N.Z. wheat cultivars has suggested that there was greater variability in *o*-diphenol oxidase activity than in phenolic content (fig. 2.11). Mean seed dry weights for these cultivars varied over a narrow range of 34.5-37.6 mg/seed. The measured parameters showed similar trends and statistical significance when expressed on a dry weight or per seed basis. Because content by weight is more relevant to milling and baking results are presented in this form.

Total phenol content varied between 286 and 554 $\mu g.g^{-1}$ and flavanol content between 12.9 and 46.2 μgg^{-1} on a dry weight basis. *O*-DPO activity showed a range of 9.90-41.05 $mV.min^{-1}.g^{-1}$ fresh weight. Analysis of variance of the three variables indicated significant differences between cultivars but no significant variability due to extract or assay replicates (table 2.4).

Comparison of cultivar o-diphenol oxidase activities has revealed trends similar to those observed in earlier studies. The relatively high levels of activity in cultivars Kotare and Otane have been observed in other lines derived from the CIMMYT breeding programme in Mexico [62],whilst the low level of activity in the white-grained cv Weka was observed to be a general feature of white wheats by Lamkin *et al*[88].

Differences in phenolic content were less marked and did not appear to parallel *o*-diphenol oxidase activity. Notably, cv Oroua showed high levels of phenolics and flavanols but low *o*-diphenol oxidase activity. Singh and Sheoran[62] observed an apparent marked correlation between browning, phenolic content and *o*-diphenol oxidase activity in their comparison of several wheats of CIMMYT and more traditional parentage. As noted in Sec.2.2 the results obtained by using such simple qualitative assays for phenolics

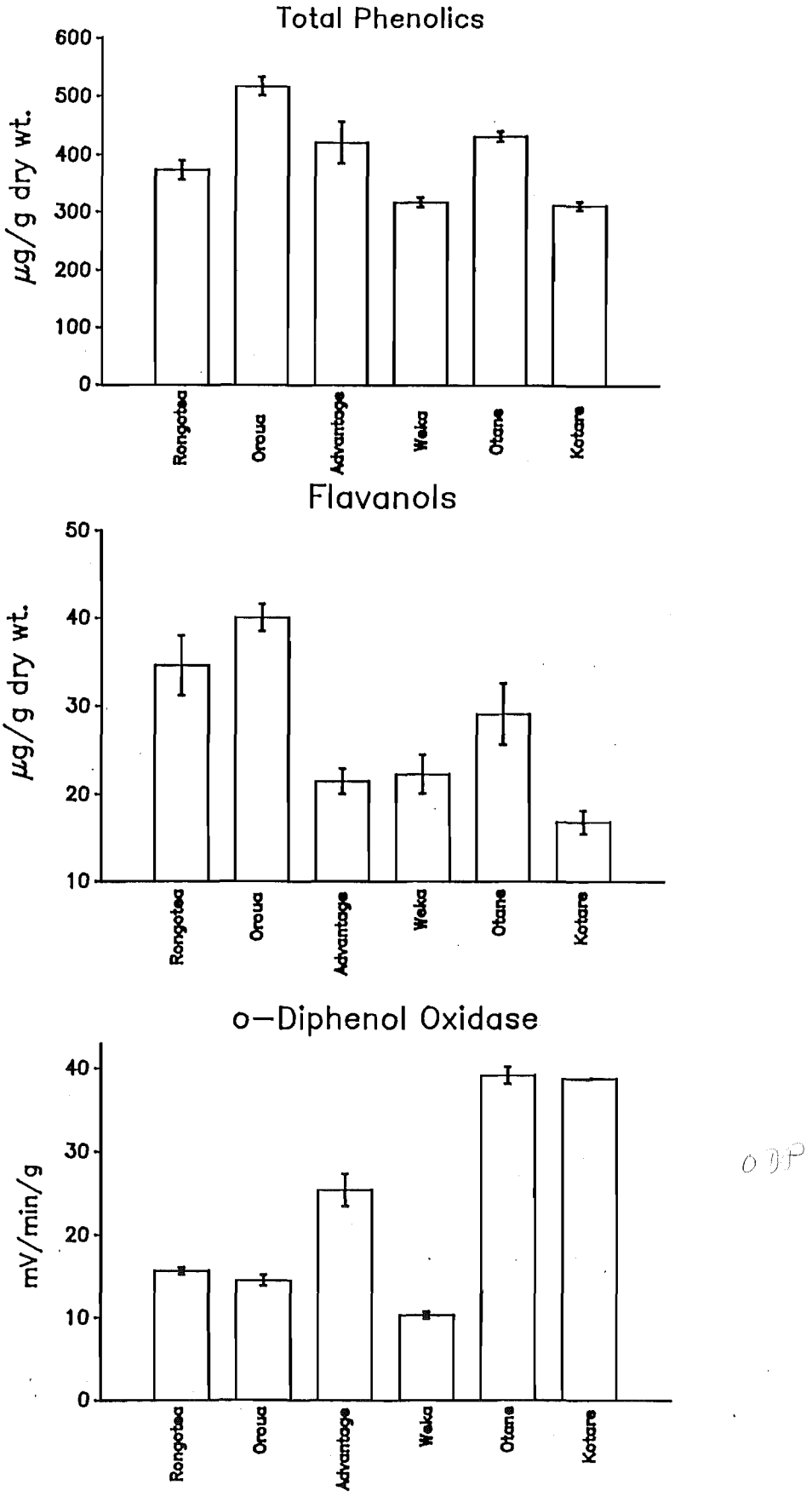


Figure 2.11 Soluble Phenolics and o-DPO Activity (Dry wt.basis) in 6 N.Z. Wheat Cvs
(Points represent mean ± std. error for n=2 extracts/ n=2 assays for phenolics; n=3 assays for o-DPO.)

must be interpreted with caution until the nature of the compounds with which they react is better understood; this is particularly the case in analysis of wheat grain where concentrations of phenolics are probably low relative to interfering substances . In a study of the relationship between seedcoat colour and dormancy in developing wheats Gordon[92] observed no relationship between these and flavanol content as determined by this assay. In view of the reactions observed noted in Section 3.3 between vanillin reagent and a variety of other compounds this assay cannot be regarded as giving a reliable indication of flavanol content *per se*.

There is further examination of *o*-diphenol oxidase activity in N.Z. wheats in Sec.2.4

Table 2.4 Comparison of Soluble Phenolics and *O*-diphenol Oxidase Activity in 6 N.Z. Wheat Cultivars

(Values are means for n=3 oxidase determinations and n=2 extracts/n=2 assays for phenols.)

Cultivar	Total Phenols (μg/g dry wt.)	Flavanols (μg/g dry wt.)	*O*-diphenol Oxidase activity (mV/min/g fresh wt.)
Rongotea	373 *bc*†	34.6 *a*	15.7 *c*
Oroua	517 *a*	40.0 *ab*	14.5 *c*
Advantage	421 *b*	21.5 *cd*	25.4 *b*
Weka	318 *c*	22.3 *cd*	10.4 *c*
Otane	432 *b*	29.1 *bc*	39.2 *a*
Kotare	312 *c*	16.9 *d*	38.7 *a*
ANOVA Results‡			
Source			
Cultivar	0.00	0.00	0.00
Extract	0.19	0.08	-
Assay	0.57	0.92	0.90

‡ Values represent p>F (the probability of obtaining a larger F-value by chance alone).

† Means followed by the same letter are not significantly different at the 5% level by a Bonferroni Pairwise comparison of means.

Coefficients of variation for analyses (extraction and assay variablity) were: Total phenols 11.4% Flavanols 21.2% *O*-diphenol Oxidase 7.9%.

2.4 *O*-Diphenol Oxidase Activity, Flour Colour and Bread Colour of NZ Wheat Cultivars.

2.4.1 Materials and Methods

Samples of bulked grain of 8 cultivars from the 1987 harvest held by Wheat Research Institute, D.S.I.R. were used in this study. Moisture content was measured by near-IR reflectance.

Grain samples (2kg) were tempered to a moisture content of 15% and milled on a Buhler experimental mill under constant conditions at an extraction rate of 72-75%. Wholemeals were prepared using a Casella blade mill fitted with a 1.5 mm screen. Flours and wholemeals were stored sealed in plastic bags at -20°C until analysis.

Measurements of flour and crumb colour, and *o*-DPO activity, and subsequent statistical analysis were performed as described in section 2.2.1.

2.4.2 Results and Discussion

The flours examined in this study showed low colour grades over a narrow range from -0.3 to 1.8 Kent-Jones units*. These low colour grades probably reflect milling at extraction rates somewhat lower than those usual in commercial mills (72 to 75% *versus* 78-79%). The flours gave correspondingly light crumb colours in the range 7.7-10.0. Flour colour and crumb colour were significantly correlated (table 2.5, fig. 2.12) but less strongly than in the mill stream flours. This may reflect the relatively narrow colour range examined.

Cv Arawa gave loaves with a slightly yellow crumb colour and the removal of this cultivar from the dataset significantly increased the correlation between flour and crumb colour from 0.63 to 0.80. These observations suggest that it may be an outlier and that factors other than the usual bran pigments are affecting its colour grade. Unlike the other six cultivars, cv Arawa is a soft wheat and it is likely that this may influence crumb colour. Unless stated otherwise cv Arawa has been retained in the data set.

A critical examination of the Kent-Jones Colour Grader by Barnes[223] has indicated that colour grade is influenced by the paste reflectance properties of the endosperm. This is likely to be most significant at low colour grades such as those observed in the present study and could account for the different behaviour of the soft wheat cv Arawa.

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*Data for all variables is tabulated in table 1 in the appendix.

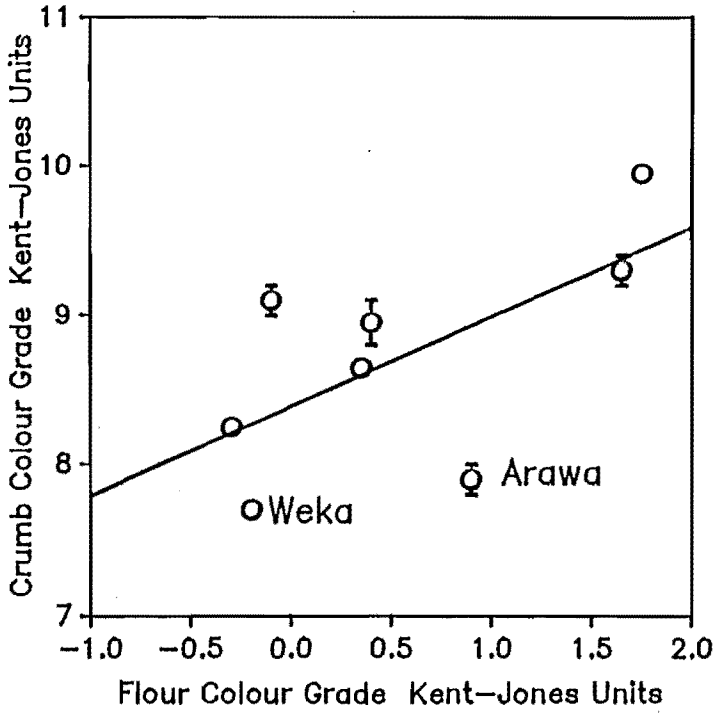

Figure 2.12 Flour and Crumb Colour Grades of 8 N.Z. Wheat Cvs. (Points represent mean ± std. error for n=2 colour grades.)

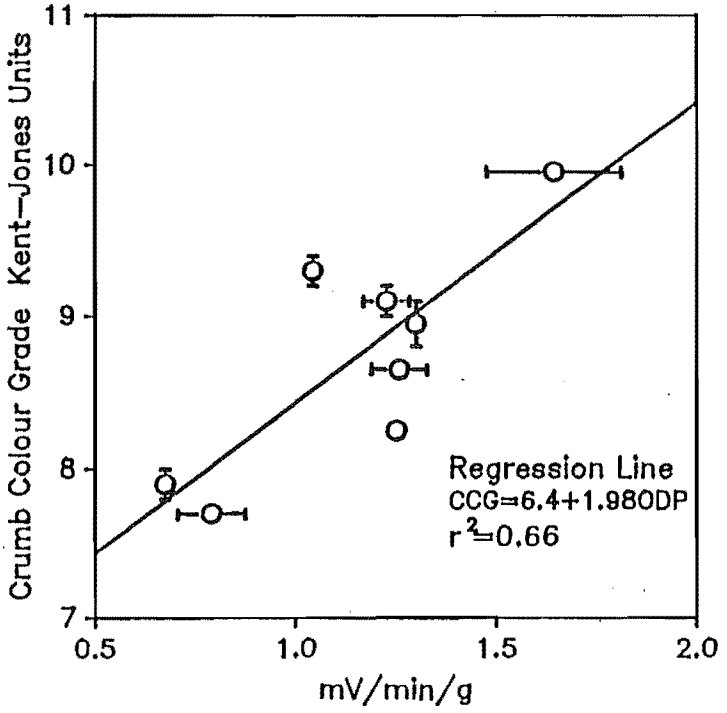

Figure 2.13 O-Diphenol Oxidase Activities and Crumb Colour Grades of 8 N.Z. Wheat cvs.(Points represent mean ± std. error for n=2 colour grades/ n=3 o-DPO assays.)

Table 2.5 Results from Correlation Analysis of Variables Measured on Flours and Wholemeals of 8 NZ Wheat Cultivars

(Values are Pearsons' Correlation Coefficient with Significance Levels, followed by n and significance p>r)

Variable	Flour Colour Grade	Crumb Colour Grade	Flour *o*-diphenol Oxidase
Crumb Colour Grade	0.636 (16,0.001)	-	
Flour *O*-Diphenol Oxidase	0.216 (48,N.S.)	0.751 (48,0.001)	-
Meal *O*-diphenol Oxidase	0.380 (48,0.01)	0.636 (48,0.001)	0.701 - (72,0.001)
Log_e Meal *O*-diphenol Oxidase	0.346 (48,0.02)	0.717* (48,0.001)	0.765* (72,0.001)

* designates corrrelation coefficient significantly higher following log_e transform.

Regression of crumb colour grade on flour colour grade (- cv Arawa) gave the following regression line:

$$CCG=8.49+0.69(FCG)\ r^2=0.65$$

the slope of which is very close to that originally reported by Pomeranz[57](0.6-0.65).

Flour colour was not significantly correlated with flour *o*-DPO activity but showed significant ($p<0.01$) correlation with wholemeal *o*-DPO activity. This suggests that wholemeal *o*-DPO activity may be a useful predictor of flour colour and that there may be a relationship between *o*-DPO activity and bran pigmentation. As observed in these (section 2.3.2) and other[88] studies, the white-grained cultivars Weka and Arawa exhibited lower *o*-DPO activities than did red cultivars[88]. Bran pigments are generally considered to be produced by the action of *o*-DPO on flavanols[38,92]. The correlation coefficients of both flour and meal *o*-DPO with flour colour grades were significantly increased (to 0.395 and 0.534 respectively) by removing cv Arawa from the data set. The nature of the yellow pigment which imparts a yellow colour to the flour of cv Arawa is as yet unknown, although it is assumed to be a xanthophyll.

Both flour and wholemeal *o*-DPO activities were correlated significantly with crumb colour (fig. 2.13). Log_e transformation of meal *o*-diphenol oxidase activity increased the correlation significantly, which suggested that this variable may not be normally distributed and that there may be a non-linear relationship between it and crumb colour.

Comparison with Data from Section 2.2.2

The relationships observed in this study between flour colour, crumb colour and flour *o*-DPO activity were compared with those observed in the study of milling flour streams, in order to determine whether these may be more general phenomena. Correlation coefficients for individual data sets were compared with those from a combined data set and the slopes of the regression lines were also compared (Table 2.5)*.

The correlation between flour and crumb colour grade was significantly decreased by combining the datasets ($p>Z=0.001$). However when cv Arawa was removed from the data set the correlation coefficients were not significantly different ($p>Z=0.06$). Comparison of the regression slopes also showed no significant difference between these ($p>0.10$). The similarity in the two distributions that this indicated suggests strongly that this relationship may hold good for most flours. Barnes[223] has suggested that flour colour grade cannot be regarded as an accurate indicator of bran content when comparing flours from different wheats due to the effects of endosperm reflectance properties. The present work, however, suggests that flour colour grade may provide a useful prediction of colour grade for flours from hard wheat cultivars.

When the corresponding statistics for crumb colour grade and *o*-DPO were examined in this manner the correlation coefficient was found to be significantly lower for the pooled data($p<0.005$) and the regression slopes were significantly different ($p<0.0005$). This latter observation does not support the idea of a causal relationship between *o*-DPO activity and normal bread crumb colour. However it cannot be ruled out that this difference may have arisen from variations in milling or assay procedures or between the two experiments, which were conducted separately and some time apart. However the significant correlations observed between *o*-DPO activity and colour in both studies does suggest that, if not *o*-DPO itself, a component or components which share the same distribution (i.e. in the bran) may be responsible for the colouration.

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* It should be noted that the correlation coefficient r generally decreases as n,the number of values, increases and therefore when using this approach there is some risk of detecting an apparent difference where there is none (i.e. type 1 error).

Table 2.6 Comparison of Relationships in Mill Stream and Cultivar Studies

1) Effect on Correlation Coefficient of Merging Datasets

Tests of the null hypothesis that merging the datasets does not change the correlation coefficient (H_0:r_1=r_2)

a) Flour and Crumb Colour Grades

Mill Streams r_1=0.98 n=12 z_1=2.32
Merged Data-Mill Stream and Cultivar Results (-Arawa) r_2=0.97 n=40 z_2=2.11
D=1.52 p>D=0.06
⇒ **Coefficients are not significantly different**
(Do not reject H_0)

b) o-Diphenol Oxidase and Crumb Colour Grades

Mill Streams r_1=0.935 n=24 z_1=1.70
Merged Data-Mill Stream and Cultivar Results r_2=0.821 df=72 z_2=1.16
D=8.63 p>D=0.005
⇒ **Coefficients are significantly different**
(Reject H_0)

2) Comparison of Regression Slopes

Tests of the null hypothesis that regression slopes are the same (H_0:b_1=b_2)

a) Flour and Crumb Colour Grade
Mill Streams CCG=8.00+0.89(FCG) SE_{slope}=0.056 n=12
Cultivars(-Arawa) CCG=8.49+0.69(FC) SE_{slope}=0.100 n=28
T=0.51 :t_{38df}=2.02
⇒ **Coefficients are not significantly different**
(Do not reject H_0)

b) *o*-Diphenol oxidase activity and crumb colour grade
Mill Stream Data CCG=7.56+14.1(ODP) SE_{slope}=1.14 n=24
Cultivar Data CCG=6.73+8.70(ODP) SE_{slope}=1.13 n=48

T=3.59 :t_{70df}=1.98
⇒ **Coefficients are significantly different**
(Reject H_0)

2.5 Conclusions

The present work and other phytochemical studies of cereals suggest that wheat grain contains relatively low levels of oxidizable phenolics[18]. In particular there appear to be little if any *o*-diphenols present which could act as substrates for *o*-DPO. Maga and Lorenz[22] have reported the presence in wheat flours of acid-labile forms of chlorogenic and isochlorogenic acids. Subsequent workers[21] were able to detect traces of alkali-labile caffeic acid (3,4-dihydroxycinnamic acid) but no chlorogenic acid. In the present studies (see section 4.2.2) chlorogenic acid was observed to be present at the milk stage of grain development using PC methods, but could not be detected in mature grain. The only other *o*-diphenols to have been reported from mature wheat grain are (+)catechin and the PAs detected in this study (see sec. 3.2.2), which also appear to be present only in trace quantities. These and other studies of grain maturation suggest that grain maturation in wheat is accompanied by a marked decline in oxidizable substances including soluble phenolics (see sec 4.2.2, 4.3.2), flavanols[92] and ascorbic acid[139]. This is presumably due to the breakdown of cellular structure that accompanies dessication in the maturing seedcoat, which would allow oxidizing enzymes and their substrates to come into contact[154].

In view of the lack of substrates for enzymic browning reactions catalysed by *o*-DPO it is therefore necessary to consider alternative mechanisms whereby browning could occur and how phenolics could participate in these.

Qualitative examination by PC has suggested that the predominant soluble phenolics in mature wheat are C-glycosyl-flavones and MHQ-triglucoside. The C-glycosyl-flavones are apigenin derivatives with a 3'-OH B-ring hydroxylation pattern and are therefore not readily oxidized. MHQ-triglucoside is not readily oxidized but the MHQ aglycone is unstable in air and readily oxidizes to methoxy-*p*-benzoquinone. Wheat germ[140] and bakers yeast[141] both contain β-glucosidase that could catalyse hydrolysis of MHQ-glucoside to MHQ under the pH conditions prevailing in a yeasted dough. The subsequent oxidation of MHQ to methoxy-*p*-benzoquinone can be catalysed by peroxidase[142]. Wheat germ[79], wheat flour[80] and bakers yeast[141] all contain peroxidase actvity which could accelerate the rate of MHQ oxidation and consequent production of coloured products. Work by Kobrehel and co-workers[60] showed that *o*-DPO and POD activities in flours were significantly correlated and that both are significantly correlated with flour paste browning. In early studies of wheat grain oxidases[72] a close correlation was observed between o-phenylenediamine oxidase and pyrogallol oxidase activities.

These observations suggest that there may be an inter-relationship between the various oxidase activities and that POD activities could be responsible for some of the enzymic browning reactions previously attributed to *o*-DPO. The oxidation of phenols such as MHQ can also be catalysed by traces amounts of iron or copper species[33,49].

O-DPO activity has been implicated in browning of chapatis by the use of inhibitors and because of apparent correlations between *o*-DPO activity (often determined by unreliable methods involving extraction and spectrophotometry [74]) and subjectively assessed dough ball browning. The inhibitors used in such studies have been either reducing agents or chelating agents, which could equally have inhibited POD activity. Peroxidative oxidation of phenols has been frequently mistaken for *o*-DPO activity, though the distinction can be readily made with appropriate assay methods[52].

There is evidence to suggest that phenolics and oxidases may participate in coupled oxidations as cofactors. C-glycosyl-flavones have been shown to act as non-obligatory cofactors for wheat leaf glycolate oxidase *in vitro*[160,161]. Oxidation of IAA *in vitro* by wheat leaf POD preparations has been shown to require the presence of monophenols or *m*-phenols as cofactors[162]. On the basis of their early work at WRI Hullett and Stern[65] suggested that in darkening of germ doughs an oxidase forms peroxide which in turn is utilised by a peroxidase to oxidize a phenolic substrate. They obtained evidence that H_2O_2 is the limiting factor in formation of colour and that oxidation of added pyrogallol by POD occurred in preference to breakdown of H_2O_2 by germ catalase[183]. During attempts to isolate the phenolics involved (MHQ had not yet been characterised) they obtained two fractions from dialysed germ extracts following precipitation with lead acetate, both of which were essential for darkening. One fraction contained C-glycosyl-flavones which might suggest that these could be involved in such an oxidase/peroxidase system as substrates or cofactors. Amine oxidases are the most common enzymes of this type[166] and therefore the presence of o-phenylenediamine oxidase activity[70] and aminophenols[40] in wheat grain also supports the idea of such an oxidase/peroxidase system.

It has long been known that doughs which have "run out of sugar" may produce a pink crumb colour[67] and in their early work Hullett and Stern noted that dough colouration was associated with a change from reducing to oxidizing conditions. These observations suggest that availability of sugars for fermentation may affect colouration through its influence on NAD(P)H/NAD(P)$^+$ ratios. Overall reducing conditions would be expected to inhibit colouration by reducing quinones non-enzymatically[169] or *via* quinone reductases[166] back to the quinol form. The addition of bromate and ascorbate during breadmaking by the MDD process would be expected to have a marked effect on

the redox properties of a dough. This fundamental difference, coupled with the shorter time taken to achieve dough maturity in the MDD process, may account for the generally lower susceptibility to dark crumb in breadmaking by this method compared to bulk fermentation methods (R.W. Cawley pers.comm.).

It is likely that wheat grain phenolics contribute to the colour of baked wheaten products by non-enzymic reactions such as condensation with furfurals produced during baking from pentosans present in flour[49].

It is also possible that there are as yet uncharacterised pigments present in some wheat cultivars which can contribute to flour and bread colour. The nature of the yellow pigment present in cv Arawa has not been established. It has been reported that addition of aqueous extracts from bran in breadmaking gives a yellow crumb colour[148].

Oxidizing and reducing agents have a marked influence on flour baking quality through their reaction with thiol groups in gluten[158]. MHQ[67] and POD/H_2O_2/catechol[159] have been shown to act as improvers and it is possible that other grain phenolics and oxidases, alone or in combination, could behave similarly.

The content of phenolics and enzymes in milling grists and flours may be markedly influenced by a variety of factors, which could result in unexpected problems of discolouration. In particular, the common practice of blending cultivars for milling could give rise to widely varying levels and proportions of these components in the resulting flour. Other environmental and physiological factors may also be of importance. In a study of English bread wheats Farrand[221] noted a strong correlation between flour colour grade and rainfall during the growing season also that poor colour grade was associated with higher protein levels. The chemical basis for the poor colour grade and its association with protein content were not examined but this work suggests that climatic factors may have a marked influence on discolouration of wheaten products.

Although intervarietal differences in enzyme activity appear to be generally greater[155] it appears that in some cases significant differences can also arise due to environmental factors[115,156]. The activity and composition of wheat grain POD has been found to be markedly affected by ripening conditions[157].

The decline in phenolics observed during grain aturation and post-harvest in this (see sec 4.2.2, 4.3.2) and other studies may influence levels of phenolics in flours milled soon after harvest. Dark crumb has usually been encountered more frequently early in the season (R.W. Cawley pers.comm.).

Phenolic biosynthesis in wheat and other plants is known to be markedly influenced by environmental factors and disease. Manganese deficiency and high nitrate levels have been shown to result in decreased levels of phenols and lignin in shoots of

young wheat plants[149]. In contrast, calcium deficiency has been shown to result in increased levels of phenolics[150]. Infection with brown rust has also been shown to result in accumulation of phenolics in wheat shoots[151].

In conclusion, the present work has confirmed that there is considerable variation among N.Z. wheat cultivars both in their phenolic content and *o*-diphenol oxidase activity. Flour colour grade has been found to be a good predictor of bread crumb colour in both milling streams and in flours from different wheat cultivars. Phenolic content and *o*-diphenol oxidase activity of a series of milling flour streams have been shown to be closely related to the colour of the flour and the crumb colour of bread baked from it and a similar relationship has been demonstrated between *o*-diphenol oxidase activity and colour of flours and bread from different wheat cultivars.

Experiments such as these, based on correlation, can provide evidence to suggest causal associations but they can never confirm them. Confirmation that phenolics and phenol oxidases are involved in colouration and discolouration of wheaten products will require a considerably better understanding of the basic chemistry of the phenolics and oxidase systems present in wheat grain.

SECTION 3-PROANTHOCYANIDINS IN WHEAT BRAN

3.1 Introduction

3.1.1 Phytochemistry of Proanthocyanidins-General

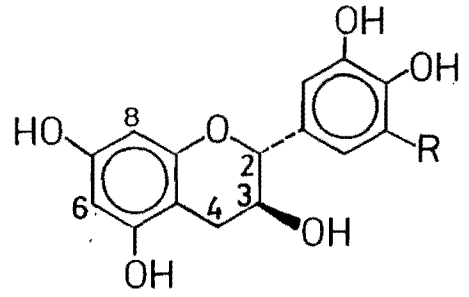
Proanthocyanidins (PAs)are colourless phenolic oligomers or polymers based on flavan-3-ol units. As the name suggests their most conspicuous property is partial conversion to anthocyanidin on acid treatment (fig3.3), a property they share with the related monomeric flavan-3,4-diols or leucoanthocyanidins[94]. The most characteristic and biologically significant property of polyphenols such as the polymeric PAs is their ability to cross-link and precipitate protein. For this reason they have also been known as "condensed tannins". PAs are widely distributed in vascular plants but are particularly common in plants of woody habit.

They consist of dimeric to high molecular weight polymeric chains of C(4)→C(8) linked 2,3-*trans*- and 2,3-*cis* flavan-3-ol units (figs. 3.1, 3.2). In some cases there may be some C(4)→C(6) linkages which permit branching of polymers. The hydroxylation pattern of the A-ring is usually 5,7-hydroxy and that of the B-ring is generally either 3',4'-OH (a procyanidin or PC unit) or 3',4',5'-OH (prodelphinidin or PD unit)[96]. As in other classes of plant biochemical there appears to be a trend towards polymer biosynthesis and the polymers are more biologically significant. Due to their high degree of hydroxylation PAs are very polar and readily form insoluble complexes with protein and carbohydrate by hydrogen bonding[145]. Although they are usually colourless PAs are highly susceptible to enzymic or air oxidation to form oxidized complexes known as "phlobaphenes". These modified PAs are less soluble and more coloured than the native PAs and are typically found in wood extractives and seed coats[95].

Flavan-3-ols and PAs have been shown to have a variety of effects on plant growth and development such as determining the germinability and vigour of lettuce seeds[164].

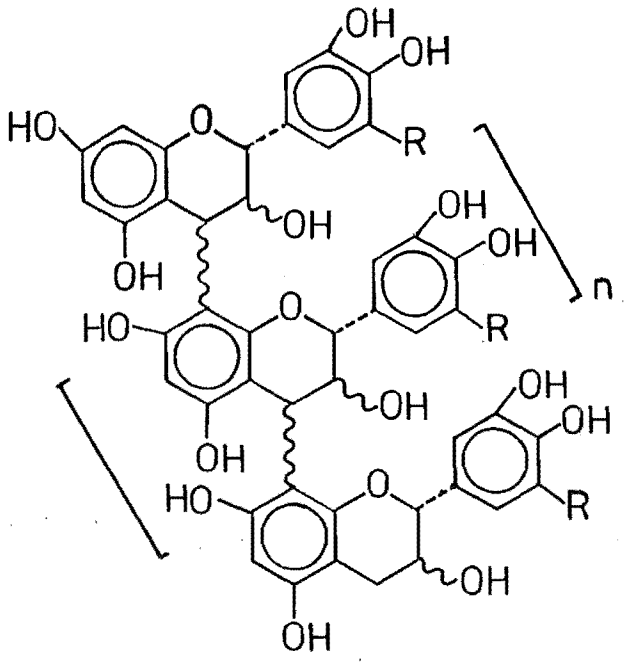
3.1.2 Proanthocyanidins in Wheat, Barley and Other Cereals: Their Significance in Cereal Seed Physiology and Food Processing

Although cereal grasses are not generally thought of as tanniniferous species their seed coats appear to typically contain a variety of flavonoids including PAs[19]. These have been found to have significant effects on the quality of several cereal grains.



R = H Catechin
R = OH Gallocatechin

Figure 3.1 Generalised Structure of a 2,3-trans Flavan-3-ol Monomer. Epicatechin and Epigallocatechin have the same hydroxylation patterns but with 2,3-*cis* configuration.



R = H Procyanidin (PC) Units
R = OH Prodelphinidin (PD) Units
n = 1 to 10.

Figure 3.2 Generalised Structure of a Proanthocyanidin Oligomer.

Sorghum typically contains high levels (up to 5% dry weight) of proanthocyanidin polymers[97] and these may significantly reduce nutritional quality in some cultivars. Recent work has indicated that in addition to PAs sorghum seed contains a variety of related compounds including glucosylated flavanoids and polymeric flavanoids containing flavanol or chalcone units[98].

Barley has been shown to contain the monomeric flavan-3-ols (+)catechin and (+)gallocatechin and a variety of procyanidin and prodelpinidin oligomers[97,98]. These have been shown to affect the colloidal stability of beer[100]. Extensive research at the Carlsberg Laboratories led to an understanding of PA biosynthesis in barley grain and production of PA-free barley varieties giving beers with excellant haze stability[101]. Histological studies of the seedcoat of developing barley grains have provided some evidence that PAs in the seedcoat may play an important role in restricting entry of water and oxygen and may thus influence germinability[222]. *In vitro* studies have suggested that PAs could also affect germinability by inhibiting giberellin-stimulated α-amylase release in barley aleurone[215].

PAs have been reported to occur in the pericarp of immature seed of brown and red rice varieties. It was suggested that the seedcoat pigments in the mature grains were phlobaphenes formed by oxidation of the PAs[102].

A similar observation to the above has also been made in immature wheat grain[38] and it was observed that there was an apparent correlation between concentration of PAs and mature seedcoat colour. It was therefore suggested that the well-known association between grain redness and dormancy was due to inhibitory effects of the PAs. This hypothesis was challenged by Gordon[92] who examined changes in flavanol content during grain maturation and failed to observe any relationship between flavanol content and dormancy Both of these studies used colorimetric methods to determine the presence and content of these compounds and no isolation of specific compounds or other validation of assays was performed. In another study utilising colorimetric methods the PA content of several cereal grains were compared and no PAs were detected in mature wheat grain[103].

3.2 Materials and Methods

Materials

Coarse commercial bran milled from cvs Rongotea and Oroua was supplied by D.H.Brown & Son,Flour Millers,Christchurch. All solvents were distilled in all-glass apparatus before use. Delphinidin, pelargonidin chloride and (-)epicatechin were

obtained from Fluka AG (Switzerland). Purified quince procyanidin was a kind gift of Dr. L.J. Porter, Chemistry Division,D.S.I.R., Petone. (+)Catechin was obtained from Sigma Chemicals (U.S.A.). Phloroglucinol, vanillin and ascorbic acid were obtained from B.D.H. Ltd..All other reagents were AR grade.

Methods

Analytical TLC was performed on Schleicher and Schull Avicel cellulose sheets. Paper chromatography of anthocyanidins was performed in descending mode on Toyo 51A chromatography paper. Solvent systems used were: **A** (TBA) *t*-butanol/glacial acetic acid/water 3:1:1. **B** (HAc)6% v/v acetic acid in water. **C** (Forestal) conc.HCl/glacial acetic acid/water 3:30:10. Flavanols were visualised by spraying chromatograms with a vanillin-HCl spray (4% vanillin in ethanol/conc. HCl 4:1) followed by warming[106].

Bran (600 grams) was ground on a Casella blade mill to pass a 1.5mm screen and percolated with 4 litres of 70% aqueous acetone containing 0.1% ascorbic acid. Combined extracts were clarified by filtration through Toyo GF paper and fractionated according to previously published methods[104,105] (fig. 3.4).

The filtered extracts were saturated with sodium chloride and the upper acetone phase was removed. This was then extracted with three successive 250ml portions of the aqueous phase of NaCl-saturated 70% aq. acetone+0.1% ascorbate. Acetone was then removed from the acetone phase by rotary evaporation at 25°C and an equal volume of water was added. This was then extracted with 3 successive portions of petroleum ether 40°-60° followed by three successive portions of ethyl acetate. Combined ethyl acetate extracts were dried with anhydrous sodium sulphate and reduced to dryness at 25°C *in vacuo*. The aqueous phase remaining after ethyl acetate extraction was dialysed in the dark with N_2 purging against three succesive 5 litre portions of 0.1% ascorbic acid and then freeze-dried.

The ethyl acetate fraction was further fractionated by chromatography on a 100 cmx2.5 cm column of Sephadex LH-20 eluted with ethanol. 50ml fractions were collected and their flavanol content was examined by cellulose TLC in solvent **B** followed by vanillin/HCl spray.

The aqueous fraction was further purified by adsorption chromatography on Sephadex LH-20. The freeze-dried dialysed extract was redissolved in 30% aq. methanol containing 0.1% ascorbate and this was applied to a 16mmx150mm column of LH-20 equilibrated with the same solvent. The column was eluted with a further 1.5 litres of 30% aq. methanol containing 0.1% ascorbate and PAs were then eluted with 200ml of

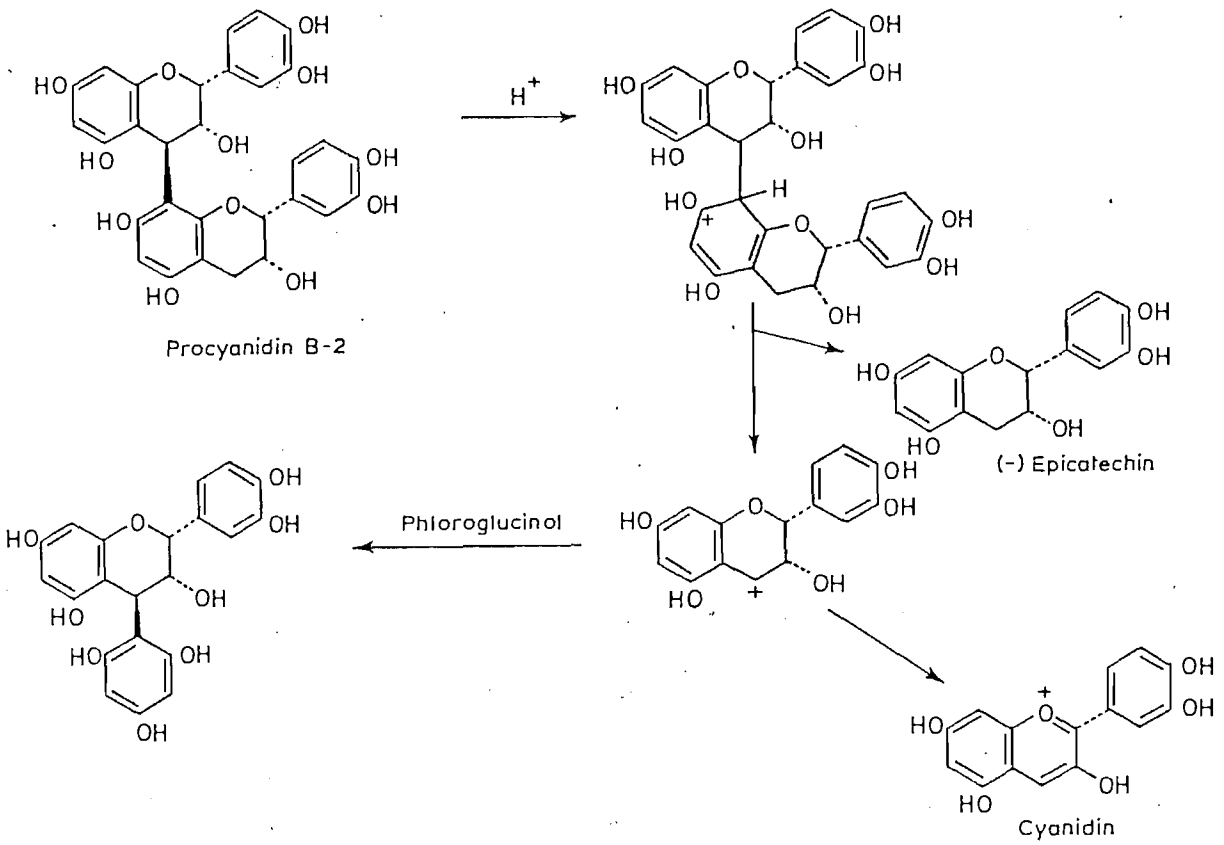


Figure 3.3 Solvolytic Reactions of Proanthocyanidins-Reactions of Procyanidin B3 (from Haslam[94]).

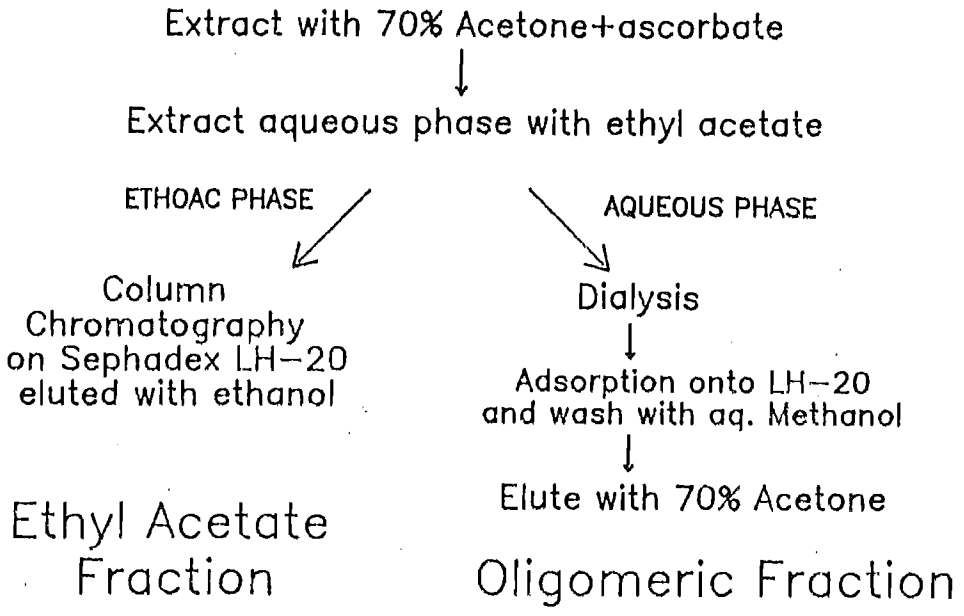


Figure 3.4 General Scheme For Isolation of Proanthocyanidins.

70% aq. acetone. Acetone was removed at 25°C *in vacuo* and the resulting aqueous residue was freeze-dried.

Acid hydrolysis of fractions and purified compounds was performed in screw-capped tubes according to standard methods[84]. Samples in methanol (0.5 ml) were heated with 3 ml *n*-butanol/conc.HCl 95:5 in a boiling water bath for 30 minutes. For chromatographic inspection hydrolysates were then dried in vacuo at 60°C, taken up in methanol containing 1% v/v conc. HCl.and separated by PC or TLC in solvent system **C** with authentic delphinidin and pelargonidin and quince procyanidin hydrolysate.

For identification of anthocyanidins separated by PC spots from hydrolysates and authentic anthocyanidins were cut out and absorption spectra were recorded directly against paper blanks in a Pye Unicam SP1800 double beam spectrophotometer. For determination of PC/PD ratios spots corresponding to cyanidin and delphinidin were cut out from PCs and eluted with two portions of methanol containing 1% conc. HCl. Extracts were concentrated *in vacuo* and cyanidin and delphinidin were estimated in the extracts by measuring absorbance at 550nm and 560nm respectively.

Acid Degradation of PAs in Presence of Phloroglucinol

Samples of PAs were incubated with an equal weight of phloroglucinol (usually 500μg each) at room temperature in 200 μl 0.1M HCl in methanol[97]. Aliquots of the reaction mixture (10μl) were removed at 0,5,15 and 30 minute intervals and after 24 hours. These were examined by cellulose TLC in solvents **A** and **B**. The original unhydrolysed material and authentic standards of (+)catechin and (-)epicatechin were similarly chromatographed. Hydrolysis products were identified by their mobility relative to standards according to data kindly supplied by Dr. L.J. Porter.

3.3 Results and Discussion

Hydrolysis of both ethyl acetate and oligomeric fractions from bran extracts in butanol/HCl suggested the presence of PAs. The visible spectra of the hydrolysates (fig. 3.5) showed maxima at 535nm for the ethyl acetate fraction and 552nm for the oligomeric fraction, suggesting the presence of anthocyanidins.

Determining yield of anthocyanidins by visible spectroscopy following hydrolysis in butanol/HCl has long been used as a means for detecting and estimating PAs in crude plant extracts[107]. Attempts to detect PAs in crude extracts of wheat grain by this method yielded brown products with visible absorbance maximum in the region of 450nm. Hydrolysis of purified MHQ triglucoside in butanol/HCl by this method also yielded products with visible absorbance maximum around 440-450nm (fig. 3.5). It is therefore

suggested that the failure to detect PAs in crude wheat extracts, in this and other studies[103],using this standard method, may be due to interference by MHQ glucosides. These are relatively abundant in wheat grain compared to other plant sources[33]. Due to their high polarity and low molecular weight they were removed by phase partitioning and dialysis during preparation of bran fractions in this study and thus detection of PAs was possible.

Analysis of the hydrolysis products by cellulose TLC in forestal solvent confirmed the presence of anthocyanidins. Hydrolysis of the ethyl acetate fraction yielded largely pelargonidin and cyanidin, along with a lesser amount of delphinidin. In contrast, the oligomeric fraction yielded predominantly delphinidin and a lesser amount of cyanidin (Plate 3.1). The identities of these anthocyanidins were confirmed by co-chromatography with authentic standards and comparison of the visible spectra of spots cut from paper chromatograms of hydrolysates with those of chromatographed standards and with previously reported data[108](Table 3.1). The PD-PC ratio of the aqueous fraction was estimated to be approximately 70:30.

Table 3.1 Forestal PC and Spectrophotometric Identification of Anthocyanidins Produced on Acid Hydrolysis of Wheat Extracts

Authentic Standards	**Rf**	**Vis.max**	
Pelargonodin	75	529nm	
Quince Procyanidin Hydrolysate	54	546nm	
Delphinidin	36	555nm	
Samples			**Probable Identity**
Ethyl Acetate	69	530nm	Pelargonidin
Fraction	50	545nm	Cyanidin
Oligomeric	51	545nm	Cyanidin
Fraction	33	555nm	Delphinidin

Cellulose TLC examination of the ethyl acetate fraction revealed the presence of at least three compounds whose chromatographic behaviour and reaction with vanillin-HCl reagent was consistent with their being (+)catechin and PAs (fig 3.6). Other compounds were also present which exhibited high Rfs in both TBA and 6% acetic acid and reacted strongly with vanillin to give red and purple colours.

Similar TLC examination of the oligomeric fraction after purification on LH-20 showed the presence of one relatively discrete spot and a diffuse streak with little mobility in 6% acetic acid (fig. 3.7). Such chromatographic behaviour suggested that these are a PA trimer and higher oligomers.

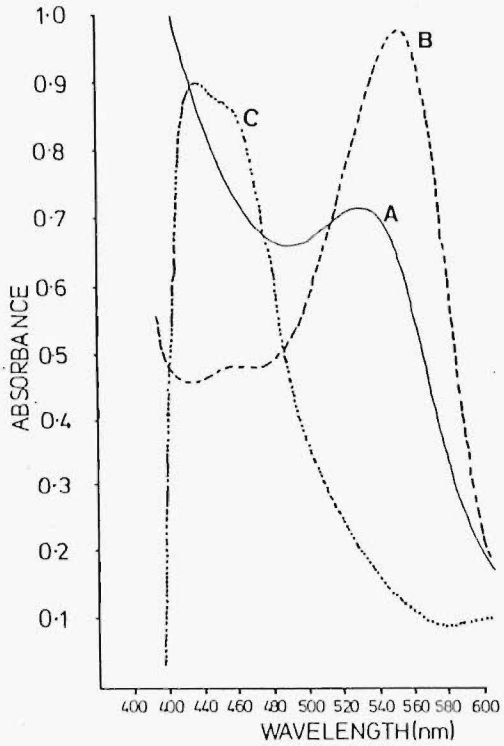
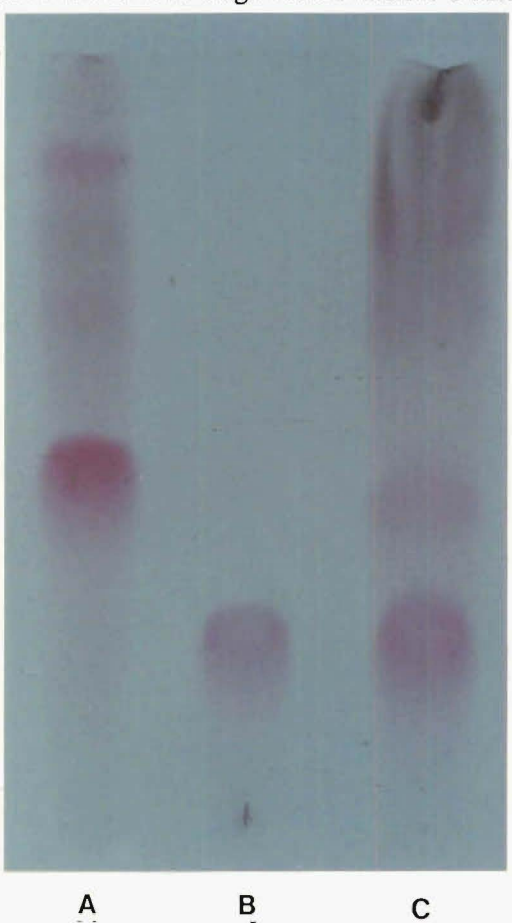
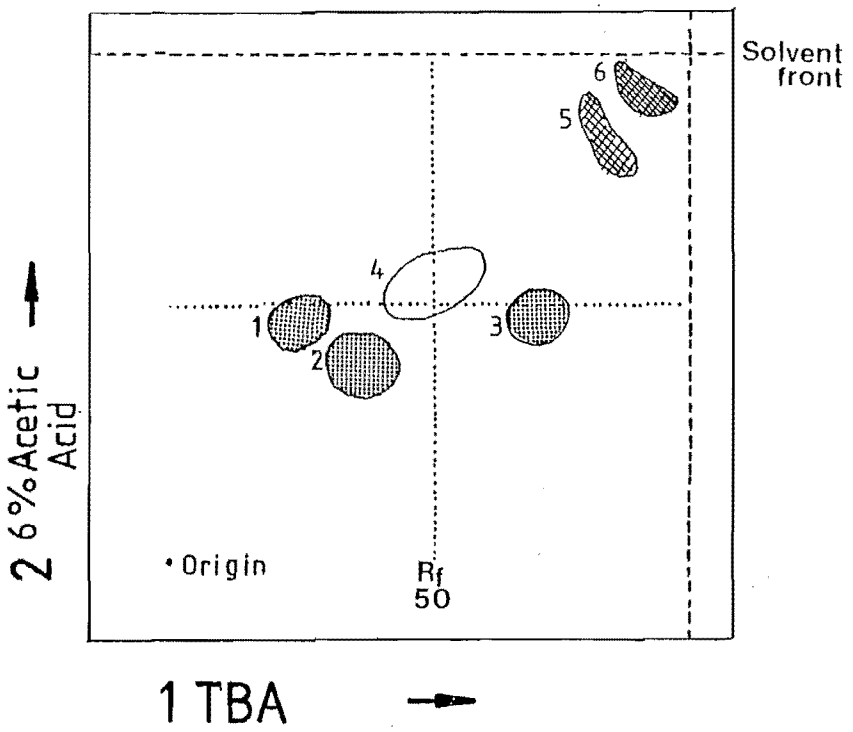


Figure 3.5 Visible Spectra of Wheat Extracts Hydrolysed in Butanol/HCl. **A** Ethyl Acetate Fraction. **B** Oligomeric Fraction. **C** MHQ triglucoside.



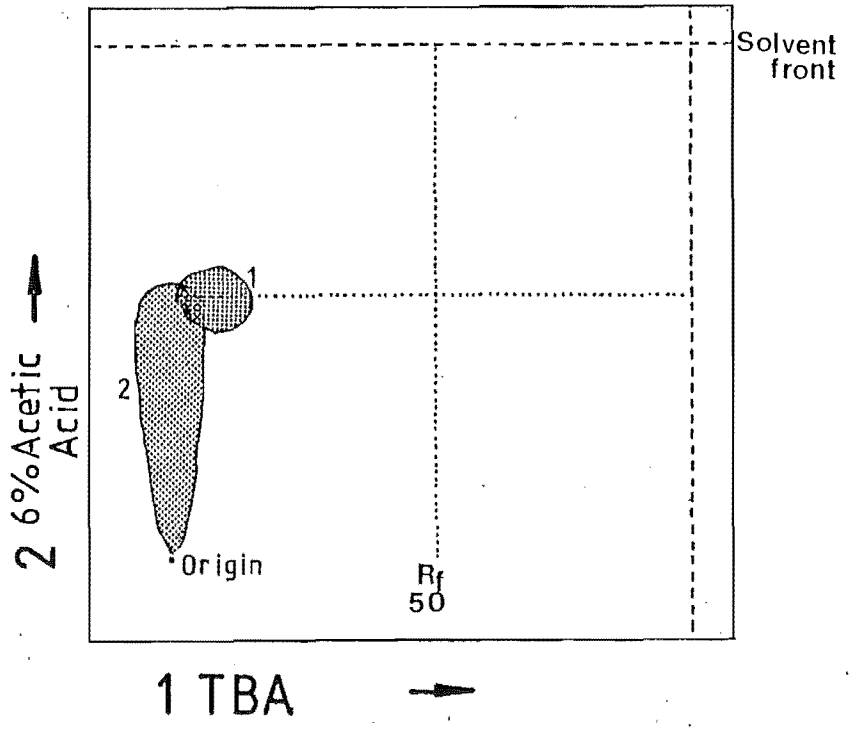
A Quince Procyanidin Hydrolysate
B Delphinidin Standard
C Wheat Oligomeric Fraction Hydrolsate.

Plate 3.1. Cellulose TLC in Forestal Solvent of Products of Acid Hydrolysis of Wheat Oligomeric PA Fraction. (Natural Colours)



1&2 PA Dimers
3 (+)catechin
4 C-glycosyl-flavone(Visible under UV)
5&6 Unknown compounds reacting with vanillin/HCl

Figure 3.6. Cellulose TLC of Ethyl Acetate Fraction



1 PA trimer
2 Higher MW PA oligomers

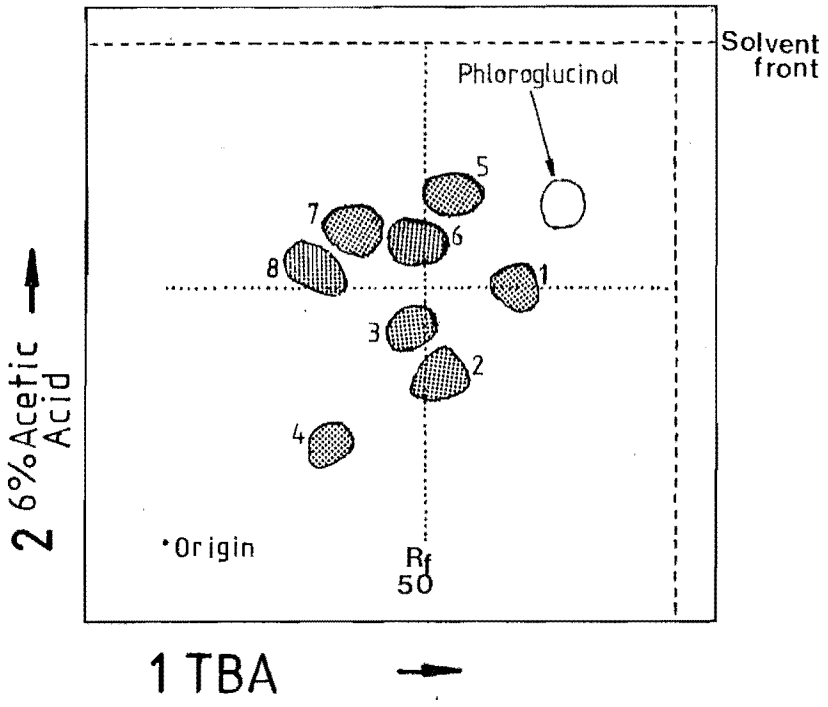
Figure 3.7 Cellulose TLC of Oligomeric Fraction.

The PAs present in the ethyl acetate fraction were further examined by column chromatography on Sephadex LH-20 with ethanol as eluant. Fractions 6-11 contained a variety of phenolics and two compounds with high mobility on cellulose in aqueous and alcoholic solvents which reacted with vanillin to give dark purple and brown colours and which would, which appear to be the same high Rf compounds noted in 2D-TLC. Following the elution of most of the other phenolic material in the extract, a flavanol was eluted in fractions 15-19 which was identified as (+)catechin by co-chromatography with an authentic sample in solvent systems **A** and **B** (Rf_A=49, Rf_B=86). This was followed by two other flavanols at much larger elution volumes, which suggested a more highly hydroxylated or polymerized state relative to catechin. These were obtained in sufficient quantity (yields approx. 2mg each) by pooling fractions 36-42 (PA1) and fractions 43-51 (PA2) to partially characterize by means of acid hydrolysis in the presence of phloroglucinol (fig. 3.5). Traces of other vanillin-reactive flavonoids were detected on TLC but were not present in sufficient quantities to characterise.

PA1 (Rf_A=53, Rf_B=60) yielded cyanidin on acid hydrolysis and when this was performed in the presence of phloroglucinol the products were identified by TLC as catechin and catechin-phloroglucinol adduct (Rf_A=60, Rf_B=57, fig. 3.9). This suggested that it was a dimeric procyanidin with the structure catechin-(4α→8)-catechin, known by the trivial name of procyanidin B3[94] (fig. 3.11).

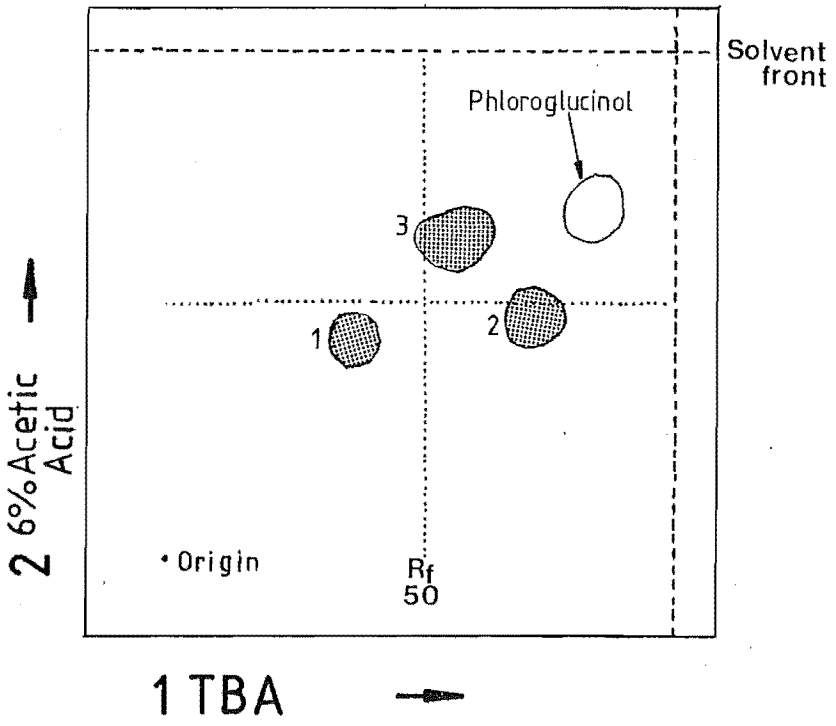
PA2 (Rf_A=42, Rf_B=57) yielded delphinidin on acidic hydrolysis and catechin and gallocatechin-phloroglucinol adduct (Rf_A=35, Rf_B=64) in the presence of phloroglucinol (fig. 3.10). This suggested that it was a dimeric prodelphinidin with the structure gallocatechin-(4α→8)-catechin (fig. 3.11). This has been known by the trivial name prodelphinidin B3[163].

Both of these dimeric PAs and (+)catechin have been reported as major components in the ethyl acetate fraction of barley phenolics[97]. These workers also reported a similar value for the PD-PC ratio in the barley oligomeric PA fraction. The failure to detect propelargonidins in the ethyl acetate extract was surprising in view of the relatively large yield of pelargonidin observed earlier on hydrolysis of such extracts. This may have been due to loss or incomplete resolution from other phenolic material during chromatography. Propelargonidins have been reported from some European malting barleys but they do not appear to be present in all barley cultivars[163].



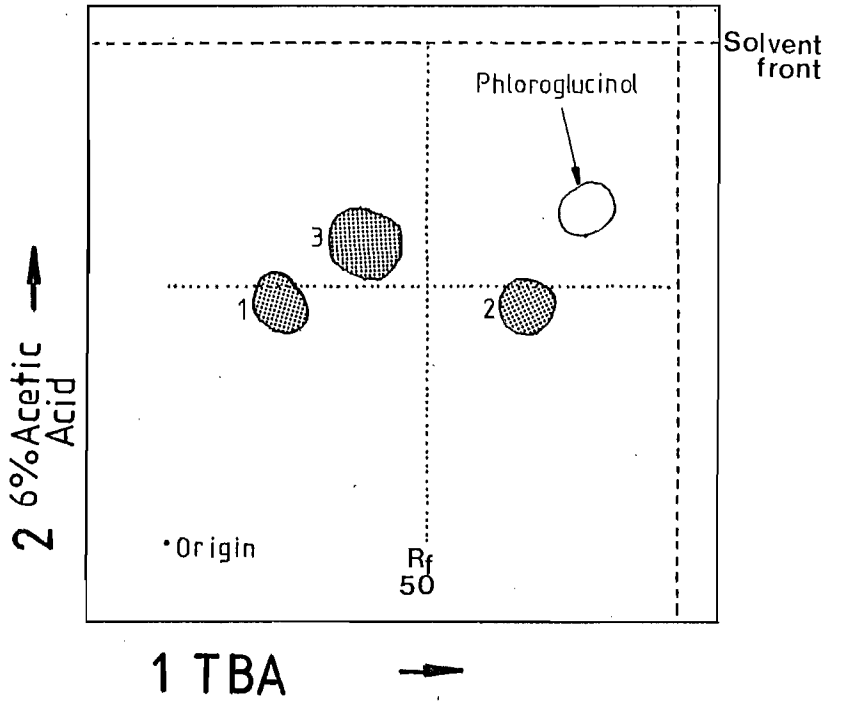
1 catechin
2 epicatechin
3 gallocatechin
4 epigallocatechin
5 catechin-phloroglucinol
6 epicatechin-phloroglucinol
7 gallocatechin-phloroglucinol
8 epigallocatechin-phloroglucinol

Figure 3.8 Cellulose TLC of Flavan-3-ols and their Phloroglucinol Adducts (supplied by Dr. L.J. Porter, D.S.I.R.).



1 Unhydrolysed PA
2 catechin
3 catechin-phloroglucinol

Figure 3.9 Cellulose TLC of Products from Hydrolysis of PA1 (procyanidin B3) in Presence of Phloroglucinol.



1 Unhydrolysed PA
2 catechin
3 gallocatechin-phloroglucinol

Figure 3.10 Cellulose TLC of Products from Hydrolysis of PA2 (Prodelphinidin B3) in Presence of Phloroglucinol.

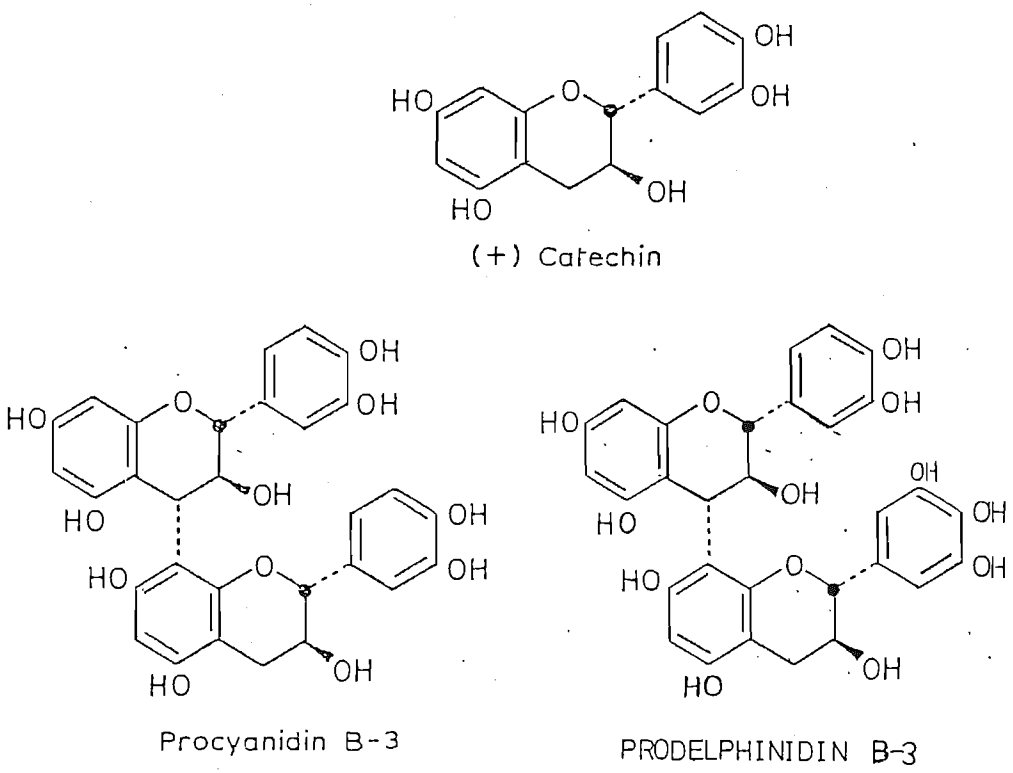


Figure 3.11 Flavan-3-ols Tentatively Identified In Wheat Bran.

3.4 Conclusions

This study has provided the first evidence that the bran of mature wheat grain contains (+)catechin and PAs. Although the amounts of extractable PAs in wheat appear to be significantly less than those reported in barley the evidence from the present study suggests that these species are qualitatively very similar. Accurate estimates of yield were difficult to obtain owing to contaminating material in the polymeric fraction and some losses during collection of fractions from the ethyl acetate fraction. On the basis of the observed yields and chromatographic evidence it is estimated that the concentration of extractable soluble catechin plus PAs was in the order of 20-40 μg/g fresh weight in this material.

Extraction of PAs from mature wheat grain is undoubtedly complicated by the presence of the various carbohydrate and protein fractions with which these compounds readily complex and the presence of active oxidative enzymes. Commonly only a proportion of PAs can be extracted from plant tissue; this may be as low as 10% in the case of polymeric prodelphinidins[49]. Enzymic oxidation and complexation is favoured by the breakdown of cellular structure that occurs in the seedcoat during grain maturation[154] and therefore it may prove more informative to study the chemistry of PAs and other wheat grain flavonoids in the "green" seed prior to dessication.

There is still a lack of information concerning the chemical nature of the seedcoat pigments of red wheats. The observation that catechin and PAs are present in wheat bran supports the original hypothesis of Miyamoto and Everson[38] that the red pigments may be formed by oxidation of these compounds. As observed in section 2.2.2, in the presence of amino acids and crude wheat grain enzyme extracts, small amounts of catechin will yield red pigments. Another important piece of evidence supporting such an origin for these pigments comes from recent work on the nature of kiwifruit skin pigmentation[109](D. Rowan and G. Lane pers.comm.). These workers observed that PAs isolated from kiwifruit skin showed a pH-sensitive colour change after oxidation but not before. They have suggested that the darkening of kiwifruit skin at high pH may be analogous to the characterisitic brick-red colours exhibited by red wheat seed coats after steeping in alkali (see plates 4.1 and 4.2). In both cases the colour changes may result from the indicator properties of oxidized PAs.

PAs could conceivably contribute to the colour of bran and wheaten products through several other mechanisms[49]. Complexation of PAs with ferric iron or copper produces strongly coloured complexes. Under weakly conditions, *in situ* formation of xanthylium salts may occur in PAs, which may also contribute to the colour of these

compounds. Furfural derivatives formed in caramelization and breakdown of sugars (notably from pentosans in wheat flour) could conceivably undergo condensation with PAs during baking to produce coloured products by reactions analogous to the vanillin reaction used to detect PAs.

PAs in the seedcoat may have particular significance in winter wheats because of their properties as protective agents. PAs are particularly resistant to microbial degradation[9] and their presence may confer some protection to overwintering seeds from soil microorganisms.

Current thinking suggests that seed dormancy is unlikely to be due to a single factor such as the concentration of PAs, but rather that there may be different types of dormancy and that these may arise through the interaction of chemical, hormonal and structural factors[198]. Recent evidence suggests that variation in response to endogenous inhibitors may be more important in determining dormancy than inhibitor concentration[110]. A study of the inheritance of germination response to PAs in excised wheat embryos suggested that this is controlled by a single gene[111]. PAs and other phenolics may therefore influence germination in some situations but our understanding of their role is limited by lack of understanding of their structures, concentration and localization in the grain.

SECTION 4-BIOSYNTHESIS AND METABOLISM OF PHENOLIC COMPOUNDS DURING WHEAT GRAIN DEVELOPMENT

4.1 Introduction

4.1.1 Development, Dormancy and Germination of Cereal Seeds

The development programme of a seed usually culminates in arrested development of the embryo, at which stage the seed can be said to be physiologically mature. A mature resting seed is said to be dormant if it is viable but does not germinate when given suitable conditions of temperature and water. A large variety of environmental and physiological factors can influence the imposition and breaking of dormancy[202]. Dormancy is genetically influenced but its expression is markedly affected by growing conditions before ripeness. Cereal breeders attempt to include sufficient dormancy to prevent pre-harvest sprouting while not adversely affecting germinability in the next growing season.

Pre-harvest sprouting of cereals is a problem of great economic significance in NZ and other areas of cereal production. Initiation of germination processes on the plant is favoured by warm, wet weather and results in variable degrees of modification of grain components due to the action of α-amylase and other enzymes[198]. These changes can render wheat unsuitable for breadmaking and other premium end-uses, with a consequently large drop in value.

Whilst seed dormancy may be an important factor in determining susceptibility to field sprouting other factors such as ear morphology and germination inhibitory factors in the bracts[201] may confer a degree of sprout resistance in some cultivars.

Three general types of germination inhibition in wheat grain have been proposed by various workers; these are: physical effects due to the pericarp limiting access of water or oxygen; inhibitors in the pericarp; and actual embryo dormancy[198]. Current thinking suggests that several factors of these types could interact to confer dormancy and that variation in response to, rather than variation in amounts of, inhibitors is more important in determining the degree of dormancy[110,111].

One of the most common approaches used by cereal breeders in NZ and elsewhere to increase dormancy has been to introduce red grain colour, which is associated with varying degrees of dormancy[203]. The NZ wheat crop currently consists principally of red-grained cultivars but there is dislike of red wheats in some markets.

Grain redness has long been known to be a quantitatively inherited characteristic controlled by up to three genes. It is more difficult to introduce sprouting resistance into white wheats, though in recent years this has been achieved with increasing success[110].

The chemical basis of the pigmentation in red wheats has been discussed in section 3.4. The initial hypothesis adavanced by Miyamoto and Everson[38] was that flavanols act as *in vivo* germination inhibitors and it was subsequently shown that exogenous PAs can inhibit germination of excised wheat embryos[205]. On the basis of colorimetric analyses of flavanols during wheat grain development, Gordon[92] suggested that this was unlikely and that oxidation of flavanols and/or the products of their oxidation may contribute to hypo-oxia in the embryo and aleurone. Apart from the equivocal evidence of Miyamoto and Everson[38] for differences in flavanol content there appears to be a lack of information concerning biochemical differences between red and white-grained wheat cultivars. Belderok[139,227] has noted differences between red and white-grained cultivars both in the structure of their seedcoats and the concentration of sulphydryl groups in these during maturation and after-ripening.

4.1.2 Biosynthesis and Metabolism of Phenolic Compounds During Cereal Seed Development and Germination

Although cereal seedlings have for long been used to study the enzymology and regulation of phenolic biosynthesis[112,113], little is known of these processes in developing seeds. The mature wheat grain contains a complex mixture of phenolic compounds derived from the pathways of phenylpropanoid and flavonoid biosynthesis and there is ample evidence that these may influence such important properties as colour, seed dormancy, resistance to fungal pathogens[19] and the physical properties of doughs and pastes[114]. A better understanding of the processes of biosynthesis and metabolism occuring during grain development and maturation may shed some light on the significance and inheritance of cereal grain phenolic content. It is conceivable that phenolics could exert an influence on the germinability or other properties of mature grain by their presence and metabolism during grain development, as well as in the mature grain[119].

There have been relatively few studies of phenolics in developing cereal seeds, doubtless due to the inconvenience of immature seeds as experimental material. Two UDP-glucose dependent phenol-glucosyltransferases have been characterised from wheat germ [175,32] and these appear to be involved in the synthesis of the MHQ-glucosides present in germ. To date these appear to be the only enzymes associated with phenolic biosynthesis to have been characterised from wheat grain.

Most studies of phenolic biosynthesis and metabolism in cereal seed development have been confined to the proanthocyanidins. Changes in concentrations of flavanols during wheat grain development have been studied using colorimetric methods[92]. Changes in sorghum[115] and barley[97] grain PAs have been examined in more detail using HPLC. Several enzymes involved in PA biosynthesis has been characterised from developing barley grain[117,118].

4.1.3 Enzymology and Regulation of Phenolic Biosynthesis

L-Phenylalanine ammonia-lyase (PAL, EC No. 4.3.1.5) catalyses the reductive deamination of L-phenylalanine to form *trans*-cinnamic acid[112], the first committed step in the biosynthesis of a variety of plant phenylpropanoid compounds including lignin, hydroxycinnamic acids and flavonoids (Fig. 4.1). Activity of this key enzyme is closely related to the physiological or developmental status of a plant and concomitant increases in levels of PAL and phenolic compounds have been demonstrated in many plant tissues[120]. In many cases this is also coordinated with the appearance of other enzymes associated with the pathways of phenylpropanoid and flavonoid biosynthesis[121]. PAL from wheat seedlings has been purified and characterised and it was observed that the purified enzyme also exhibited L-tyrosine ammonia-lyase (TAL) activity[122]. This is a characteristic of PAL from many monocot species but not dicots[120]. Recent studies suggest that PAL may occur in multiple forms with different pIs and kinetic parameters[223].

Following the initial PAL catalysed deamination a variety of cinnamic acids and their CoA esters are formed by the successive action of hydroxylases, *o*-methyl transferases and CoA ligases[123]. The CoA esters are the metabolically active form of these acids and act as substrates for subsequent synthesis of lignin, flavonoids and acyl esters.

The initial step in flavonoid biosynthesis is the condensation of hydroxycinnamoyl-CoA esters with malonyl-CoA catalysed by chalcone synthetase (CHS)[124]. This appears to be the rate-limiting step for flavonoid biosynthesis and thus another key regulatory locus; analogous to PAL[125]. The second common step in the biosynthesis of all flavonoids is the stereospecific isomerisation of the chalcone product of the initial condensation to the corresponding (-)flavanone, which is catalysed by the enzyme chalcone-flavanone isomerase (CHI) E.C. 5.5.1.6[127] fig. 4.2). The flavanone product serves as substrate for biosynthesis of the various classes of flavonoid. Some workers have observed multiple forms of CHI and recent studies suggest that there may be significant differences in the properties of enzymes from different sources[224].

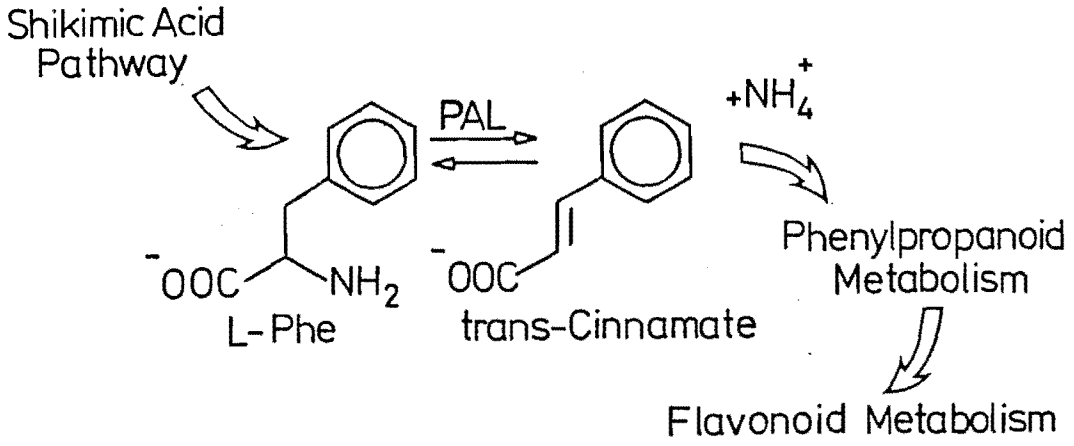


Figure 4.1 Reaction Catalyzed by L-Phenylalanine-Ammonia Lyase (PAL) and Its Relation To Plant Primary and Secondary Metabolism.

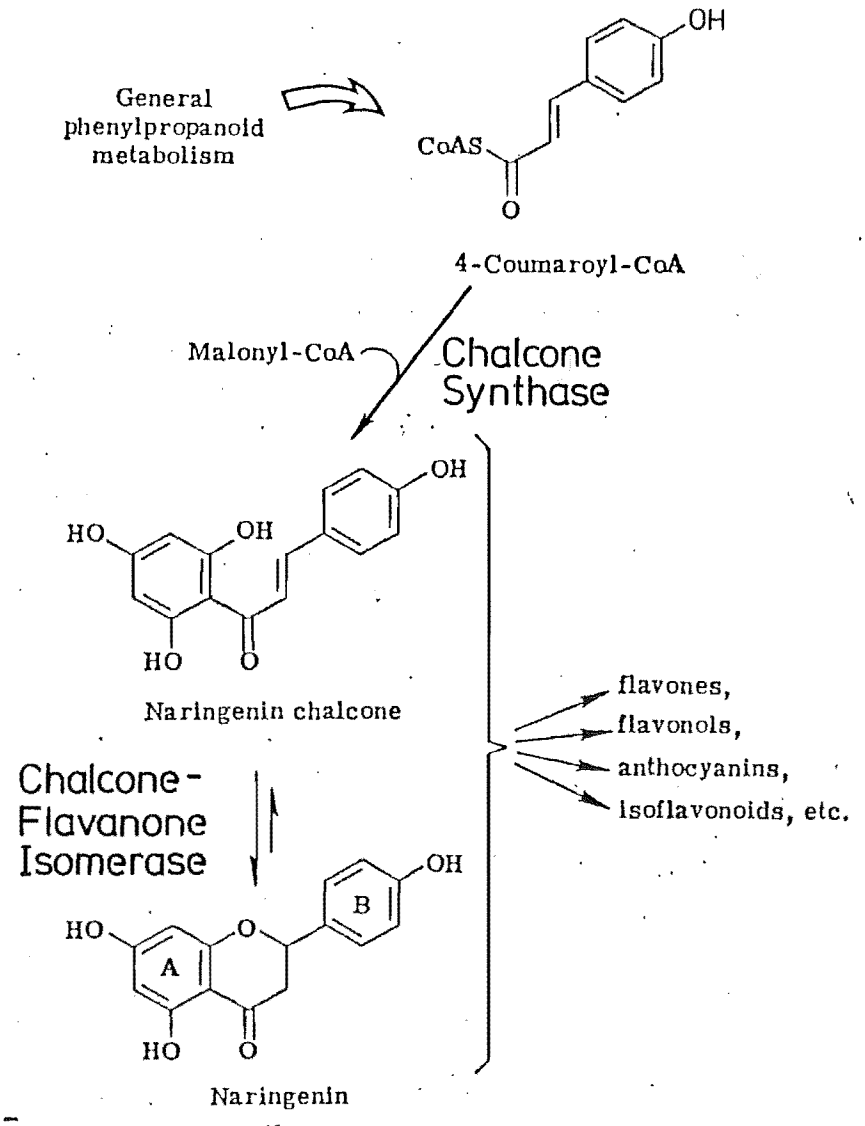


Figure 4.2 Reaction Catalyzed by Chalcone-Flavanone Isomerase (CHI) and Its Relation to Phenylpropanoid and Flavonoid Biosynthesis.

All the evidence to date suggests that phenolic biosynthesis is highly regulated and typically involves a co-ordinated short-term increase in expression of genes for individual pathway enzymes to give bursts of synthesis of particular end products at particular stages in the development of a plant or in response to stimuli from the environment[228]. This degree of regulation in the pathway and its receptivity toward a variety of factors strongly suggests that phenolics play an important role in the metabolism of plants and their interaction with the environment.

4.1.4 Hydroxycinnamic Acids in Cereal Seeds

Although hydroxycinnamic acids are present in wheat grain in both free and conjugated soluble forms (see Sec. 1.2, fig. 4.13) the greatest quantities occur in insoluble conjugated forms[20,21]. Ferulic and *p*-coumaric acids occur covalently bound to cell walls of the Poaceae and several other plant families[23]. Arabino-xylans from cell walls of wheat bran[127] and wheat endosperm[128] have been shown to contain ferulic acid esterified *via* non-reducing arabinofuranosyl residues. The autofluorescence of wheat aleurone and scutellar epithelial cell walls has been shown to be due to bound ferulic acid[129].

In addition to alkali-labile ester-linked forms, wheat flour[22], straw[199] and other tissues also contain acid-labile hydroxycinnamic acids. Scalbert and Monties[200] have suggested that these represent β-aryl ethers and may represent an important linkage between lignin and cell wall carbohydates. Lignin is confined to the testa-pericarp of cereal grains[195].

N-Feruloyl-glycine and -phenylalanine sequences have been reported to occur in barley seed globulins[147] and recently an N-feruloylglycine amidohydrolase has been characterised from barley embryo[148]. It has been suggested that the presence of such substitution may influence the structure and mobilization of cereal seed storage proteins.

The double bond of the cinnamic acids confers several important properties. Notably it shifts the absorbance maximum of these compounds into the UV-A region (320-400nm) whilst *cis/trans* isomerism is possible around the double bond and is catalysed by UV irradiation[130]. However hydroxycinnamic acids are synthesized and occur predominantly in the *trans* configuration. It has been suggested that UV-A mediated photoisomerism of wall-bound hydroxycinnamic acids may provide the mechanism for phototropic and "blue light" responses in plants[4]. This isomerism may also provide a mechanism for regulation of hydroxycinnamate biosynthesis by lowering levels of *trans*-cinnamate, which has multiple inhibitory effects on PAL activity[189,7]. There is evidence that the activated double bond may itself participate in the enzyme-catalyzed

formation of β,β-dilactone dimers[165], UV-catalyzed formation of truxillic acid dimers containing a cyclobutyl structure[234] or the formation of adducts with cysteine thiols[131].

Because of their bifunctional nature hydroxycinnamic acids may form both ester and ether linkages by reaction of their carboxyl and phenolic groups respectively and thus participate in the cross-linking of cell wall macromolecules[195]. Recent evidence suggests that photodimerization of wall-bound hydroxycinnamic acids could also contribute to such cross-linking[234]. It has been proposed that formation of diaryl linkages between tyrosine or ferulic acid residues, catalysed by wall-bound peroxidases, may regulate primary cell wall expansion[132]. Diferulic acid has been detected in wheat flour arabinoxylans and it is thought that the oxidative gelation of flour pentosans is due to formation of such cross-links[133]. It has been suggested that phenolic ester linkages may play a role in holding together arabinoxylans and cellulosic polymers in wheat bran cell walls[134]. Overall there is much evidence to suggest that wall-bound hydroxycinnamic acids may play an important role in regulation of the physical properties of cereal cell wall components during growth and at maturity.

4.2 1986/87 Trial

4.2.1 Materials and Methods

Materials

L-Phenylalanine (L-Phe), trifluoroacetic acid (TFA), Amberlite XAD-2 resin, gallic acid, chlorogenic acid and cinnamic acid were obtained from Sigma Chemical Co. U.S.A.. Cinnamic acid was recrystallized from aqueous ethanol before use. Trichloroacetic acid was obtained from B.D.H. Ltd.. Octadecyl silica (C-18) was obtained from the Dept. of Chemistry, University of Canterbury. HPLC grade water and methanol were used throughout. Folin-Denis reagent was prepared according to A.O.A.C. methods[81]. XAD-2 resin and polyvinylpolypyrrolidone(PVPP) were washed before use to remove UV-absorbing materials[143,144]. Phadebas tablets for α-amylase assays were obtained from Pharmacia Fine Chemical AB (Sweden).

All wheats were grown in irrigated plots at the Crop Research Division, D.S.I.R., Lincoln. Fungicide application has been reported to affect PAL activity in cereal seedlings[137]; therefore none was applied in this study.

Methods

Plot Design and Sampling

Plots of the red-grained cultivars Otane and Alcala, and of the white-grained cultivars Veery and Cook, were sown in 1.5m long strips laid out in two blocks. The date of 50% ear emergence was estimated for each cultivar as the day on which 50% of ears had become completely free of their flag leaves.

At each sampling date, one strip per cultivar was selected randomly from each block and the entire strip was cut down. Sampling was commenced about 14 days after 50% ear emergence and continued at 5-6 day intervals until harvest ripeness.

All sampling was carried out between 9am and 11am. Whole ears were frozen in liquid N_2 and stored at -20°C in double polythene bags. PAL activity was stable for at least two months under these conditions. Samples for analysis of phenols were freeze-dried and stored in double polythene bags at -20°C prior to analysis.

Moisture determination

The moisture content of 50 seed samples was estimated by weighing before and after drying at 60°C for 24h.

Extraction and Assay of PAL Activity

Grains were dissected from the outer florets of spikelets in the central part of individual spikes. Triplicate extracts were prepared from each sample.

For each extract 25 seeds were first ground in a mortar and pestle with liquid N_2 and then homogenised for 30 seconds in 10ml chilled 0.05M phosphate buffer, pH 6.6, using an Ultra-Turrax homogenizer (Janke & Kunkel F.R.G.). The suspension was filtered through two layers of Miracloth and centrifuged at 4°C for 15 minutes at 13,000g. The supernatant was decanted and passed through a 10mmx30mm column of XAD-2/PVPP(1:1) previously equilibrated with the extraction buffer. The column was washed with a further 4ml buffer and the eluates were stored on ice and used as a crude enzyme source.

PAL activity in the crude enzyme extracts was assayed by an adaptation of the methods of Zucker[135]. The assay mixture consisted of 3.5ml 0.06M borate buffer pH8.8 and 1ml crude enzyme and the reaction was initiated by the addition of 1ml 10mg/ml L-phenylalanine (final concentration 11mM). Tubes were incubated at 37°C for 1 hour. The reaction was stopped by the addition of 0.5ml 35% w/v trichloracetic acid and tubes were centrifuged for 5min at 5000G. The yield of cinnamic acid was estimated by measuring A_{290} of the supernatant in 1cm acrylic cuvettes. Triplicate assays were performed for each extract, with and without substrate, in order to compensate for increases in absorbance even in the absence of added L-Phe[136].

Protein concentrations were determined by the dye-binding method of Bradford[138].

HPLC Validation of PAL Assay

For assay of cinnamic acid production by HPLC a 500μl aliquot of the acidified reaction mixture was mixed with 200μl water and 100μl 2.5mg/ml benzoic acid in 80%aq. methanol as internal standard. This was then applied to a 5mm x 12mm column of C-18 (100mg) equilibrated with 5mM TFA. The column was washed with a further 800μl 5mM TFA and eluted with 1.6ml 80% aq. methanol, with the aid of gentle suction. The methanol eluate was dried in vacuo at 40°C, redissolved in 500μl 80% methanol and filtered through a 0.45μm PTFE filter. Duplicate 50μl samples were analysed using a Varian 5000 HPLC fitted with a Brownlee 10cm 5μm C-18 column + 1.5 cm 5μm C-18

guard column and a UV detector coupled to a Spectra-Physics computing integrator. The mobile phase was methanol:acetic acid:water/40:2.5:57.5 at a flow rate of 2 ml/min. Separations were performed at ambient temperature and detection was carried out at 280nm. Retention times and peak areas were calculated relative to the benzoic acid internal standard.

Analysis of PAL Data

The raw data was first inspected for normality using the program BMDP2D[82].

Rates were calculated for each extract from the difference of the mean absorbances with and without added L-phenylalanine. The rates were subjected to repeated-measures model ANOVAs using the program BMDP8V[82]. Seedcoat colour and sampling time were designated as fixed effects factors and cultivar, block and extract replicate as random effects.

Localization of PAL Activity

Subcellular distribution of PAL activity was examined in extracts prepared from a sample of cv Otane collected at 25/12/86 (approximately 23 days after ear emergence).

A homogenate was prepared as described above but with the addition of an equal weight of PVPP during the initial grinding in N_2. This was then centrifuged for 2 minutes at 500G and the pellet was discarded. The supernatant was then centrifuged at 15,000G for 15 minutes at 4°C, the resulting supernatant was decanted and the pellet was resuspended in the chilled extraction buffer. This supernatant was centrifuged at 100,000G for 2 hours at 4°C and the resulting pellet was resuspended as previously.

Analysis of Soluble Phenolics

Freeze-dried grain of cultivars Otane and Veery was hand cleaned and ground in a Casella blade mill to pass a 16G screen. Duplicate samples of meal equivalent to 250 seeds were extracted with three successive 50ml portions of 75%(v/v) aqueous acetone in N_2-flushed centrifuge bottles for 30 minutes. The aqueous acetone extracts were filtered off through Whatman No.1 filter paper and pooled extracts were reduced at 30°C *in vacuo* to 20% original volume. The aqueous phase was assayed in triplicate for soluble phenols using Folin-Denis reagent with gallic acid as a standard[83].

For preparation of extracts for chromatographic inspection samples were prepared as for qualitative analysis and the aqueous residue obtained was adjusted to pH 2.5 with 2M TFA. This was then applied to a 10mmx15mm column of C-18 previously equilibrated with 5mM TFA. The column was washed with further 5mM TFA and

phenolics were eluted with 80% methanol. The eluate was reduced to dryness at 40°C *in vacuo* and redissolved in a small volume of 80% methanol for chromatography. Descending paper chromatography (PC) was performed on large (46 x 57cm) sheets of Whatman 3MM paper. A quantity of extract corresponding to approximately 40-50 grains was applied to each sheet and these were developed successively with: **1** BAW (n-butanol:acetic acid:water/40:10:22); **2** 15% acetic acid.

o-Diphenol Oxidase Activity of Mature Grain

Mature grain of the four cultivars from both blocks was ground on a blade mill to Pass a 28G screen and o-DPO activity was assayed as described in section 2.2.1.

PAL and α-Amylase Activity in Germinating Grain

Samples of 25 seeds of cv Otane (mature grain obtained from this trial) were placed on two 7cm disks of Whatman #1 filter paper moistened with 2ml distilled water in Petri dishes and incubated in darkness at 25°C. Duplicate dishes were removed at 4, 21, 28 and 45 hours after imbibition commenced. After removal the seeds were frozen in liquid N_2 and stored at -20°C until analysis.

Crude enzyme extracts were prepared from samples as decribed above. PAL activity was assayed in extracts as described previously. α-amylase activity was assayed using Pharmacia Phadebas dye-labelled substrate tablets[230].

PAL Activity in Etiolated Seedlings

Mature grain obtained from the trial was germinated on moist vermiculite and grown in the dark at 25°C for seven days. The shoots were cut off at the level of the vermiculite and assayed for PAL activity as previously.

4.2.2 Results and Discussion

Timing of Growth and Development

The dates of 50% ear emergence and days from then to anthesis (in brackets) were as follows: Cultivars Veery and Alcala 1/12/86 (4); Cv Otane 2/12/86 (4); Cv Cook 5/12/86 (4).

All cultivars reached the dough stage of grain development[167] and attained mature grain colour between five and six weeks after anthesis. By 22/1/87 approximately 50% of ears of all cultivars had attained mature grain colour.

During the 1986/87 season breeders noted that cv Alcala was not readily distinguishable from cv Otane by means of the usual tests. This suggested that these cultivars had very similar parentage and therefore were not ideal for such a comparative study.

Owing to the use of bulked samples in this study there was substantial variation in degree of development within samples and this was particularly marked in early samples. Since the variation between and within ears in grain development was felt to be at least as significant as differences in timing of mean ear emergence and anthesis between cultivars the timecourse results have been presented in terms of sampling date.

Changes in moisture content and dry weight for the four cultivars are shown in figure 4.3. All cultivars had reached harvest ripeness (20% moisture or less) by 5/2/87.

Table 4.1 Grain Weight and Moisture Content of Mature Grain

Cultivar	**%moisture**	**Fresh wt.** mg/seed	**Dry wt.** mg/seed
Cook	11.7%	48.1	42.5
Veery	10.9%	50.8	45.3
Alcala	11.0%	57.1	50.8
Otane	11.7%	57.1	50.4

PAL Activity in Developing Grain

Assay of PAL Activity

Significant levels of PAL activity were found in extracts of green developing seeds during the milk stage of grain development[167]. Activity could not be conclusively detected in mature seed. TAL activity was also detected in extracts of immature seed using a spectrophotometric assay but owing to the unreliability of this assay procedure[168] it was not examined further. Neish[205] has reported the presence of TAL activity in barley ears at milk stage but could not detect it in wheatgerm.

An examination of the pH-dependance of PAL activity over the range 8.4-9.2 suggested that it had a broad optimum around ph 8.8-9.0 (fig 4.4). Similar values have been reported for the enzymes from wheat seedlings[122] and most other sources[168] and therefore all subsequent assays were performed at pH 8.8. Validation by reversed-phase HPLC (fig 4.5) confirmed that net increases in absorbance at 290nm could be accounted for by corresponding increases in the concentration of cinnamic acid. These were found to be linear for at least two hours under the conditions employed (fig 4.6).

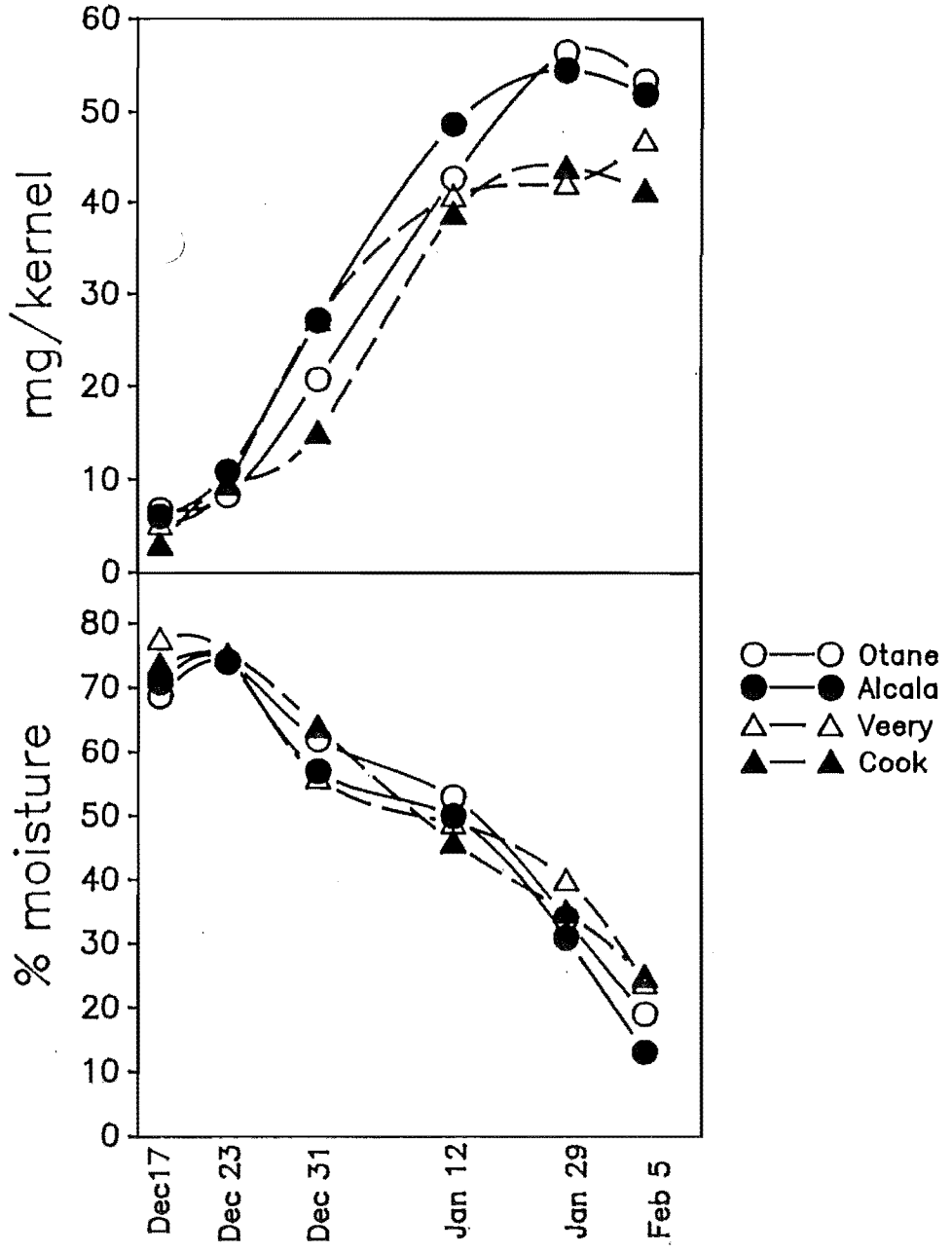


Figure 4.3 Changes in Moisture Content and Dry Weight During Grain Development of Cultivars in 1986/87 Trial (Points represent mean values from three samples of 50 seeds).

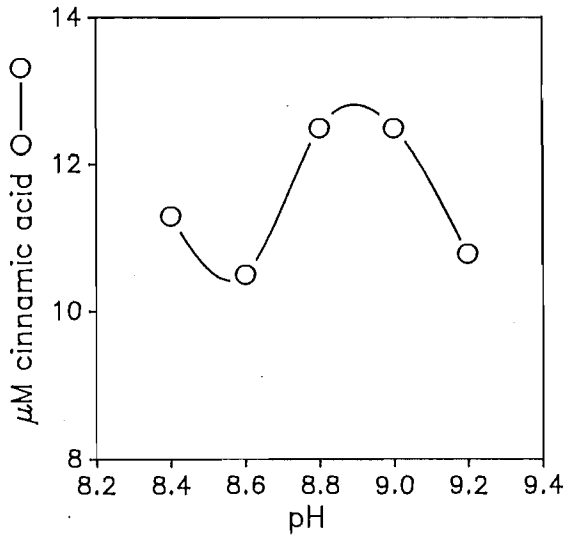


Figure 4.4 pH-Dependance of PAL Activity From Immature Wheat Seed (Points represent the mean of triplicate assays).

HPLC has not been widely used as a means of assaying PAL activity as *trans*-cinnamic acid production but it has several advantages over other methods. Because the products are separated chromatographically there is less likelihood of interference by other compounds produced or occurring in crude plant extracts[170]. The greater sensitivity of HPLC detection also means that smaller samples and shorter incubation times could be used. The solid-phase extraction step used in this study would be unnecessary if a pre-column was used. With the use of an internal standard reliable estimates of *trans*-cinnamic acid could therefore be obtained directly from 20-50 μl of the de-proteinized assay mixture.

Subcellular Localization of PAL Activity

Differential centrifugation of crude extracts suggested that most PAL activity (75%) could not be sedimented even after prolonged centrifugation at 100,000G (table 4.2). Less than 5% of the total activity was sedimented at 13,000G with chloroplast debris. This indicates that significant activity was not being lost in the centrifugation step during preparation of extracts in the normal method used for sample preparation. This small proportion of activity may represent non-specific association of enzyme with chloroplasts (and other organelles) during fractionation[206]. A significant proportion of the PAL activity was sedimented at 100,000G, suggesting a microsomal location. Much recent evidence suggests that PAL and other enzymes of phenolic biosynthesis are associated with the endoplasmic reticulum *in vivo* and that observation of "soluble" enzymes, such as in this case, are an artefact caused by the homogenization and media employed in fractionation[206].

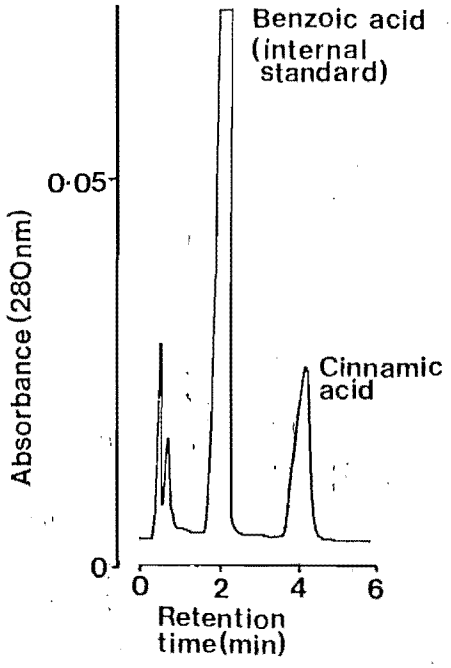


Figure 4.5 HPLC Chromatogram of Reaction Mixture Following Incubation Of Crude Wheat Seed Enzyme Extract With 11mM L-Phe for 2 Hours.

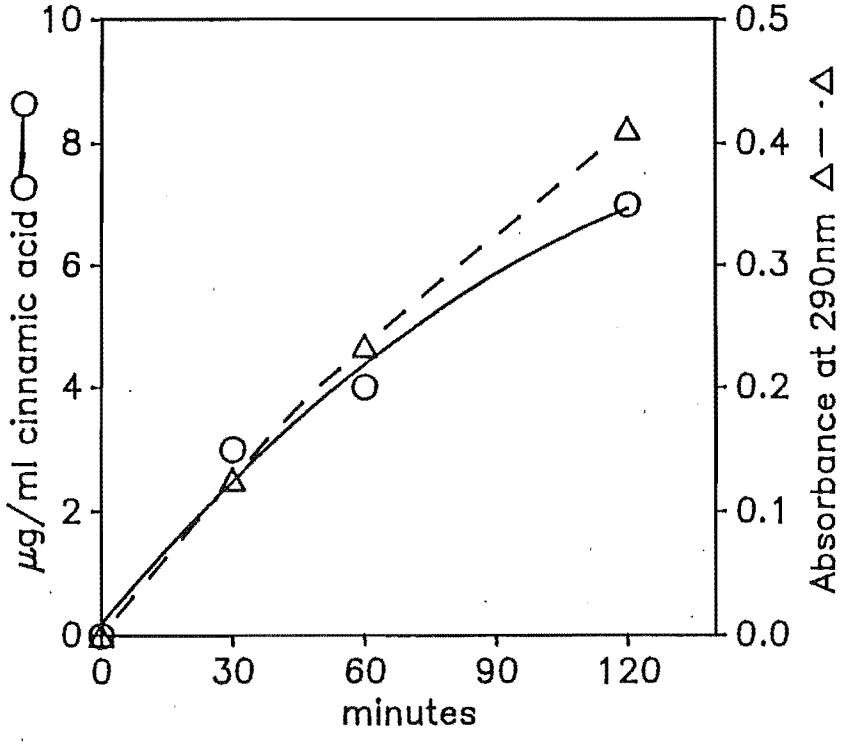


Figure 4.6 Relationship Between Net Yield of *trans*-Cinnamic Acid Estimated by HPLC and Absorbance of De-proteinized Reaction Mixture.

Table 4.2 Distribution of PAL Activity in Fractions Obtained From Differential Centrifugation of Crude Homogenates

Fraction	%Total Activity	Activity (pKat/seed)	Specific Activity (pKat/mg protein)
13,000G pellet	4.6	0.42	3.56
100,000G pellet	19.2	1.75	16.42
100,000G supernatant	76.3	6.97	19.36

(Values represent means of triplicate determinations)

Changes in PAL Activity

Assays of seed extracts from early milk to dough stage suggested that peak activity per seed occurred at around 20-30 days after ear emergence (15-25 days post-anthesis) in the four cultivars studied (fig. 4.7). This peak in activity occurred in the milk phase of grain development when moisture content was still high (55-60%). Other workers have noted that a-amylase[171] and peptide hydrolase[155] activities per seed peak around this time and that these enzyme activities are particularly high in the pericarp tissues. The subsequent decline in activity coincided with an accelerated rate of dry weight increase and the onset of endosperm solidification at soft dough stage[167]. It has been observed that α-amylase activities of developing barley seed also decline rapidly during the linear phase of grain filling[172].

When results were plotted in terms of specific activity (fig. 4.7), or as activity on a dry weight basis, less informative profiles were obtained which suggested that differences in dry weight and protein accumulation in the different cultivars were obscuring common patterns of change in PAL activity when results were re-expressed in this manner. High correlations were observed between specific activity, activity/dry weight. and moisture content (table 4.3), confirming the inverse relationship between activity and grain filling noted above.

Table 4.3 Correlation of Grain Moisture Content and PAL Activity

	Specific Activity	Activity/g Dry wt.	
Moisture Content	0.76	0.80	(n=16, $p<0.001$ for all correlations)
Specific Activity	-	0.89	

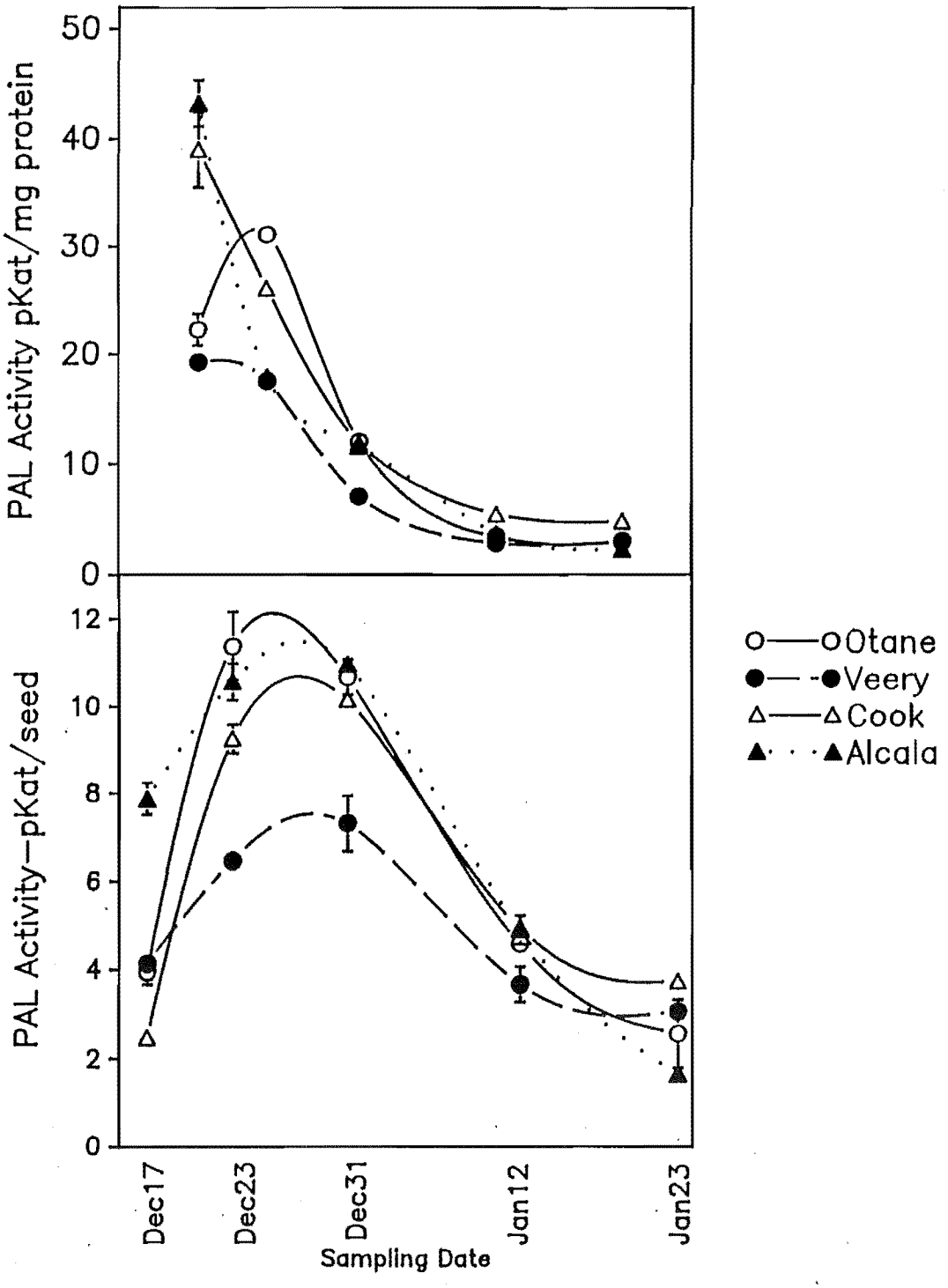


Figure 4.7 PAL Activity in Developing Grain of Four Cultivars in 1986/87 Trial.(Points represent the mean±SEM of n=3 extracts, each assayed in triplicate).

Comparison of Cultivars

The peak levels of PAL activity per seed were observed to be higher in the red-grained cultivars. ANOVA of the results, in terms of activity per seed, indicated a significant grain colour effect (table 4.4). This confirmed that PAL activity per seed was significantly higher in the red cultivars than in the white ones. However due to the small number of cultivars examined (n=2 cultivars per colour and thus only 1 degree of freedom) it was necessary to examine critically this result in order to assess its practical significance and to suggest improvements in experimental design for further trials.

Table 4.4 Estimates of Variance Components from ANOVA of 1986/7 PAL Activities/seed

Source	%contribution
colour	**12.44**
time	53.94
cultivar	**9.99**
block	2.85
extracts	0.98
colour x time	0
colour x block	0
cultivar x time	**12.33**
block x time	1.37
colour x extract	0
cultivar x extract	1.30
block x extract	1.30
cultivar x block x time	2.63
Cultivar x block x extracts	0.79 **

** coefficient of variation for determination of extract activities=4.8%

Cultivar and cultivar/time interactions were also significant, suggesting that there were also significant differences among cultivars of the same colour in the amount of PAL activity **and** in the way these changed over time. Inspection of the plotted data (fig. 4.7) suggested that the significant cultivar effect might have been due to the large difference between cultivars Veery and Cook around the time of peak PAL activity. This hypothesis was tested by repeating the ANOVA without colour as a factor before and after excluding cv Veery from the data set (table 4.5).

Table 4.5 Estimated Variance Components from ANOVA of PAL Activity/Seed Data Before and After Exclusion of Cv Veery

Source	%Contribution (+cv Veery)	%Contribution (- cv Veery)
Cultivar	**12.9**	**4.8**
Time	64.5	74.0
Blocks	1.2	0.9
Extracts	2.2	2.5
Cultivar x Time	**13.2**	**12.4**
Block x Time	3.3	2.6
Cultivar x Extract	0.3	0.3
Block x Extract(Error)	2.4	2.4

Removal of cv Veery clearly had a large effect on the estimated cultivar variance component, which suggested that most but not all of the colour effect was due to the relatively low PAL activity observed in this cultivar. However its removal did not have a significant effect on the cultivar/time effect and the magnitude of this component may in part reflect a weakness of the sampling procedure. Due to the considerable variability noted previously in the stage of development of ears in early samples the profiles of activity observed can only be considered to give a low resolution picture of changes in PAL activity in the developing grain and this creates difficulties when attempting to compare cultivars. The imprecision in defining sample age would be expected to lead to confounding and thus reduce the likelihood of detecting differences between colours by increasing the time variance component and associated interaction effects.

Phenolics in Developing Grain

Quantitative Analysis of Phenolics

Quantitative analysis of soluble phenolics in freeze-dried samples of cultivars Otane and Veery revealed similar trends in both cultivars(fig. 4.8). The peak in soluble phenolics per seed appeared to coincide with the decline in growth rate and appearance of mature grain colour. This peak also lagged behind the peak in PAL activity by 15-20 days. The content of phenols per seed in cv Otane was higher than cv Veery throughout development and this difference was found by ANOVA to be statistically significant ($p<0.001$). However the concentration of phenolics in the two cultivars, when expressed

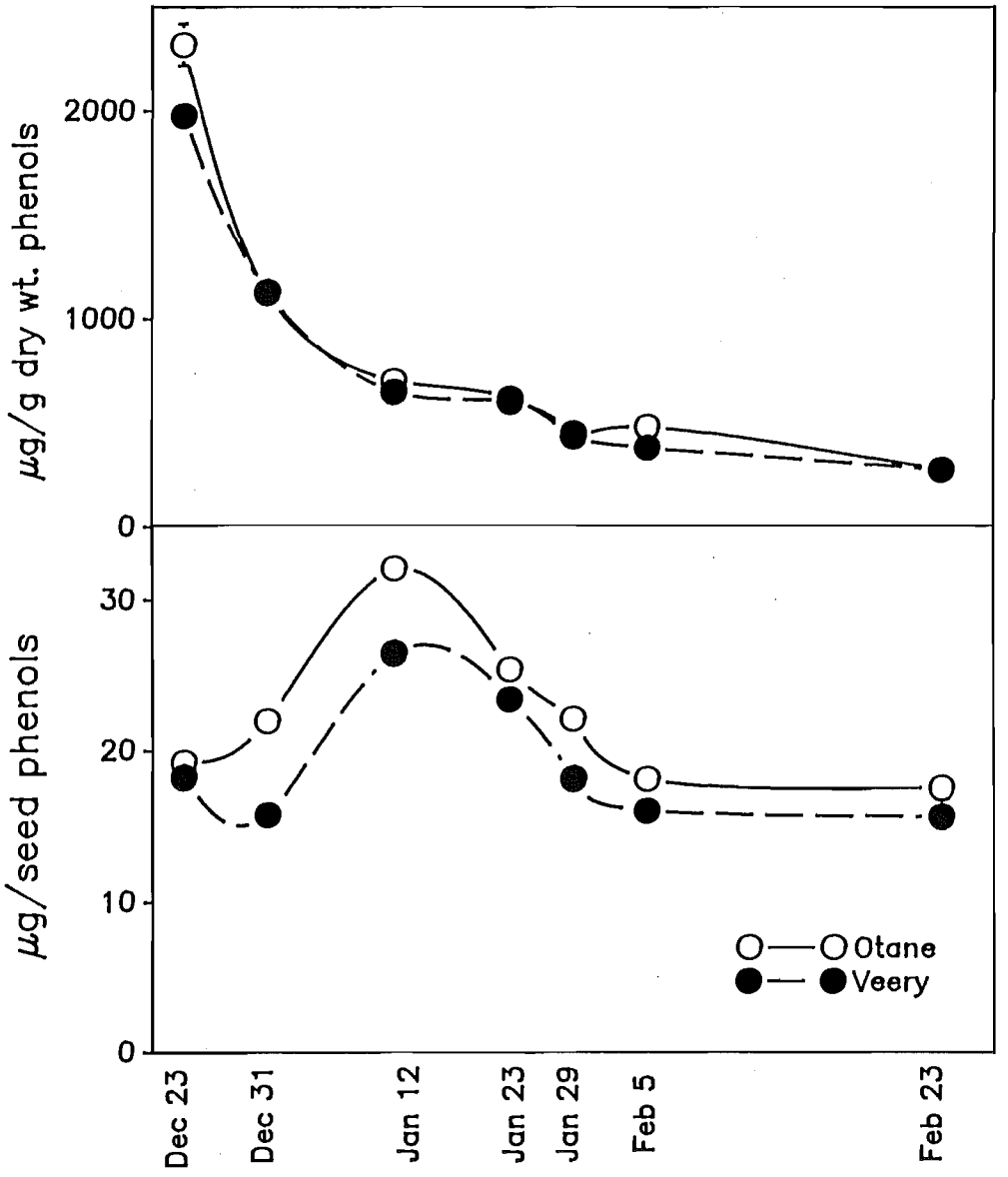


Figure 4.8 Soluble Phenolics Determined by Folin-Denis Assay in Developing Grain of cvs Otane and Veery (Points represent the mean±SEM for n=2 extracts each assayed in triplicate).

on a dry weight basis, was not found to be significantly different. Declines in the concentrations of flavanols[92], and ascorbic acid[139] during maturation have been noted during previous studies of wheat grain development. A similar pattern of changes in total phenolic content was also observed in study of developing sorghum grain[116]. Such declines in concentrations of oxidizable substances are probably a result of contact with oxidative enzymes during the breakdown in cellular structure in the seedcoat tissues that accompanies grain maturation[154]. As noted in section 2.2.2, the Folin-Denis phenols assay is prone to interference by other reducing agents and therefore these results could be more cautiously interpreted as a measure of "oxidizable substances". The fact that development of mature grain colour was associated with a marked decline in phenolics supports the idea that the products of their oxidation may be seedcoat pigments.

The auxin content in developing wheat grain has been found to exhibit a similar peak just prior to termination of dry weight gain and the development of mature grain colour[207]. Peroxidase activity also peaks around this time[208] and may be associated with metabolism of phenolics in the testa-pericarp. On the basis of studies on wheat coleptiles, Machakova *et al*[196] have suggested that degradation of phenolics by peroxidase *in vivo* may be regulated by auxin levels.

Paper Chromatography of Phenolics in Developing Grain

Samples of phenolics prepared from grain of cultivars Otane and Veery collected at late milk stage (12/1/87) and at maturity (23/2/87) were examined initially by reversed-phase HPLC. However this technique proved impractical due to the large number of UV-absorbing components present in chromatograms and the difficulty in resolving and identifying these. Two-dimensional paper chromatography (2D-PC) proved to be much more practical for qualitative examination of grain phenolics as this provided good resolution (with large sheets) as well as permitting easier identification of phenolics by their fluoresence characteristics.

No qualitative differences could be distinguished between cultivars Otane and Veery in immature or mature grain extracts. However marked qualitative and quantitative differences were evident between the phenolic content of immature and mature grain (figs. 4.9, 4.10). In general it was noted that there was a marked decline in the amounts of hydroxycinnamic acid derivatives present on maturation. In contrast there appeared to be an increase in quantities of C-glycosyl-flavones as well as qualitative changes in these compounds. Considerable qualitative and quantitaive variation in C-glycosyl-flavone content has been observed in oats between different plant parts, under differing

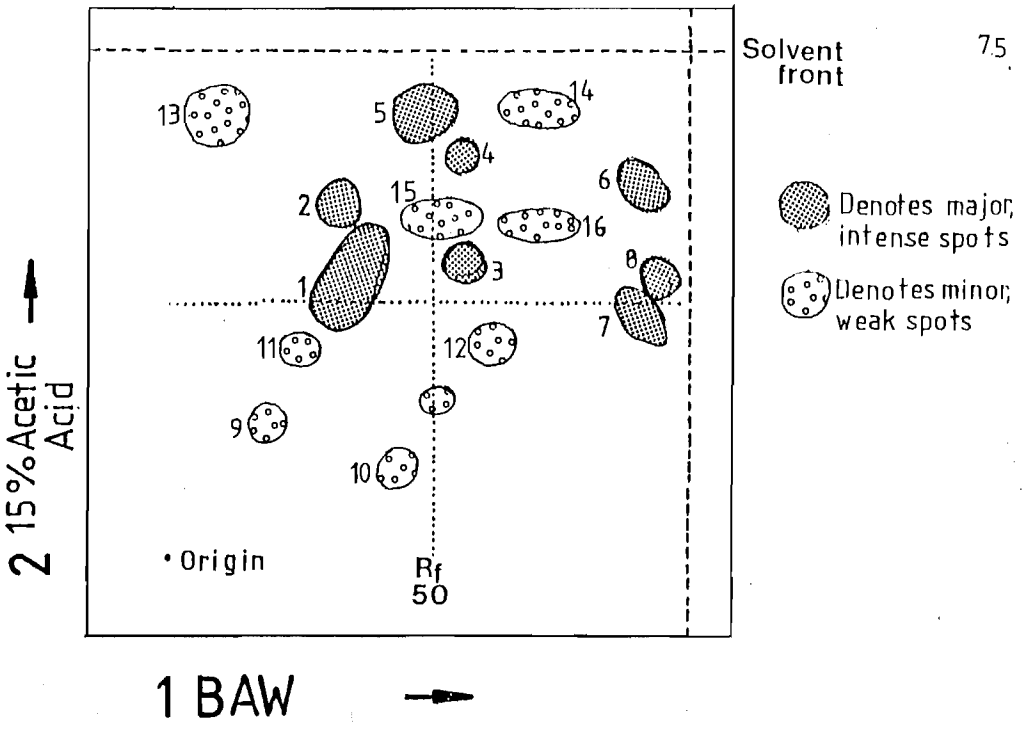


Figure 4.9 Paper Chromatography of Soluble Phenolics of cv Otane at Milk Ripe Stage

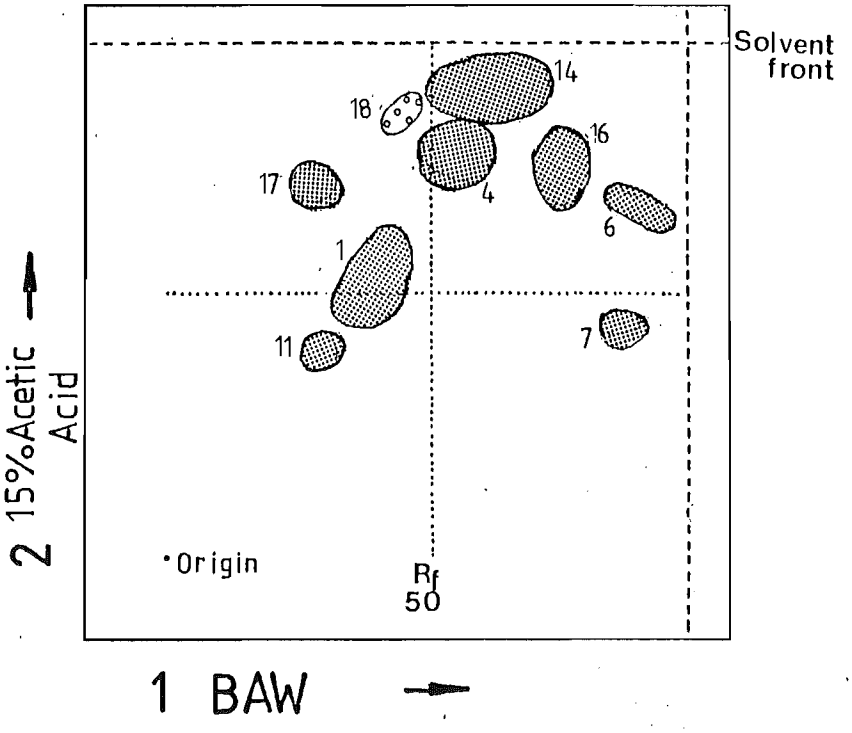


Figure 4.10 Paper Chromatography of Soluble Phenolics of cv Otane at Maturity

conditions of growth and at different stages of devlopment[19,209]

Notably, chlorogenic acid (fig. 4.11) was tentatively identified in chromatograms of immature grain by its R_f values and characteristic fluorescence properties under UV±NH_3. However it could not be detected in chromatograms of mature grain. This phenolic, found in a wide variety of taxa[1], has been previously reported to occur in wheat leaves[173], though its presence in mature grain has not been established unequivocally[21,22].

o-Diphenol Oxidase Activity of Mature Grain

The o-DPO activities of mature grain of the four cultivars exhibited trends noted earlier in cultivar surveys (Sec. 2.3.2), with activities being higher in the red-grained cultivars. ANOVA of the results from this study indicated that not only were differences between cultivars significant but that there was a significant effect due to blocks (Table 4.6). This observation suggests that although cultivar differences are more significant, significant variability within a cultivar may arise due to environmental influences.

Table 4.6 Means and ANOVA Table For *o*-Diphenol Oxidase Activities of Mature Grain.

Cultivar	Mean o-DPO Activity mV/min/g
Otane	22.80 a
Alcala	21.73 a
Veery	19.68 b
Cook	13.33 c

(Means followed by the same letter are not significantly different at p=0.05 by a Bonferroni pairwise comparison of means)

Source	DF	SS	MS	F	P>F
Cultivar	3	323.3	107.8	118.1	0.000
Block	1	9.8	9.8	10.7	0.005
Cultivar x Block	3	14.9	5.0	5.5	0.009
Error(Cultivar x Block x Assay Reps)	16	14.6	0.9		

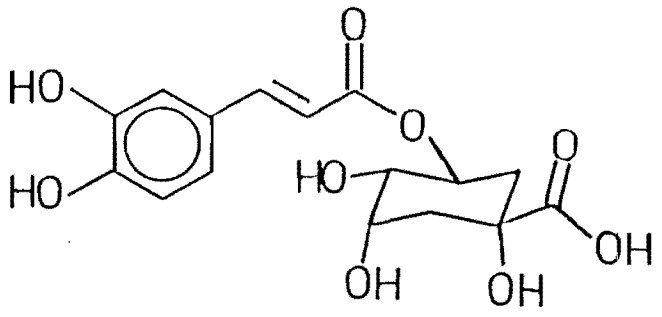


Figure 4.11 Chlorogenic Acid (Caffeoylquinic Acid).

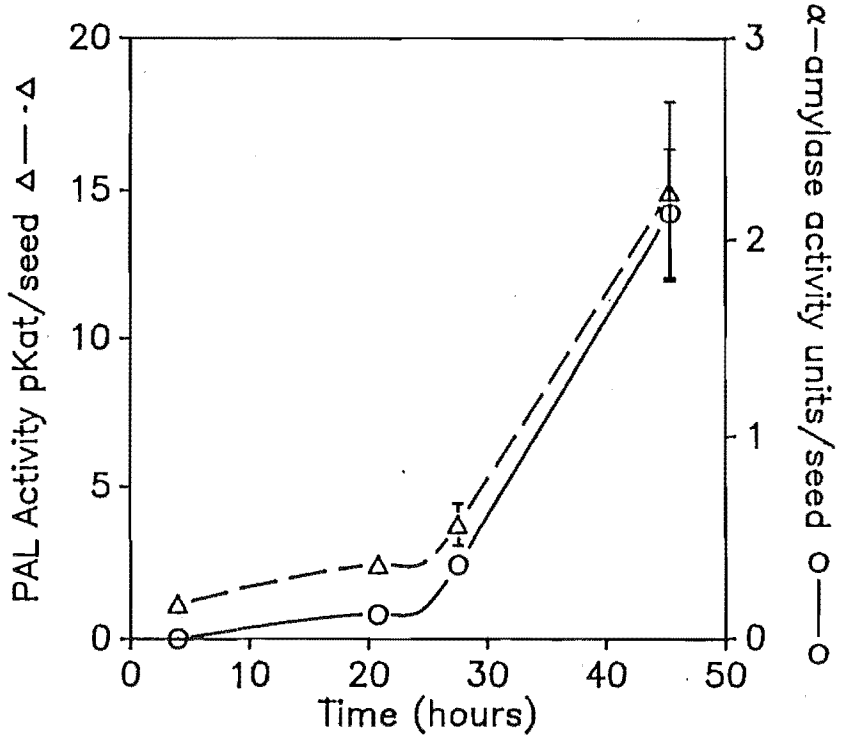


Figure 4.12 PAL and Alpha-Amylase Activity in Germinating Grain of cv Otane (points represent mean±SEM for n=2 extracts assayed in duplicate).

PAL and α-Amylase in Germinating Grain

The development of PAL and α-amylase activity were found to follow very similar timecourses in germinating grain of cv Otane (fig. 4.12). PAL activity was correlated significantly with α-amylase activity ($r=0.995$, $p<0.001$). The similarity of the timing of appearance of PAL activity observed in developing grain in this study and that of α-amylase observed in other studies has been noted already. This similarity in both development and germination suggest that both enzymes are subject to similar regulation or co-regulation. The comparatively late appearance of α-amylase during germination is a result of requirement for *de novo* synthesis from existing mRNA[119].

PAL Activity of Etiolated Seedlings

Assay of PAL activity in seedlings of the four cultivars indicated that there were significant differences between them both in the specific activity and activity on a fresh weight basis. However there was no apparent relationship between these and activity during grain development (table 4.7). It is possible that these differences may be due to different rates of germination and growth, although these did not appear to be great.

Table 4.7 PAL Activity in Etiolated Seedlings of Four Wheat Cultivars

Cultivar	PAL Activity pKat/g fresh weight	PAL Specific Activity pKat/mg protein
Otane	285*b*	57.1*a*
Alcala	183*c*	30.8*b*
Veery	158*c*	29.4*b*
Cook	401*a*	77.2*a*

(Values followed by the same letter are not significantly different at $p=0.05$ by a Bonferroni pairwise comparison of means)

Experimental Design for Subsequent Trial in 1987/88 Season

In view of the results obtained in this trial it was felt that it would be worthwhile to carry out a further trial in order to establish more conclusively whether red and white-grained cultivars differed in their PAL activities during early grain development. In view of the apparent differences in PAL activity it was also decided to examine whether there might be any differences between red and white cultivars in enzymes associated with flavonoid biosynthesis. In order to obtain more specific information on changes in phenolics during grain development than could be obtained using colorimetric assays it was decided to examine changes in hydroxycinnamic acids, in particular ferulic acid, using HPLC methods.

To test more effectively whether red and white cultivars differed in their PAL activities it was necessary to reduce variability in the sampling and it was decided to do this by tagging ears at emergence in order to accurately define age. It was planned to examine four red and four white cultivars of diverse parentage in order to provide comparisons of greater practical and statistical strength. As it was clearly desirable to obtain more detailed information on changes in the parameters of interest throughout grain development it was planned that cv Otane would be sampled and assayed throughout development. For comparative studies, all cultivars would be sampled at four times during early development over the period of maximal PAL activity observed in this initial trial.

4.3 1987/88 TRIAL

4.3.1 Materials and Methods

Materials

Amyloglucosidase, *p*-coumaric acid and naringin were obtained from Sigma Chemical Co. (U.S.A.). Celluclast, a crude cellulase and xylanase preparation, was obtained from Novo Industries Ltd.. Folin-Ciocalteau phenol reagent was obtained from B.D.H. Ltd..

Naringenin was prepared from naringin by refluxing for 2 hours in 2M HCl and allowing the mixture to cool. The crude product was filtered off and after treatment with activated charcoal and recrystallization from aqueous methanol gave crystals of naringenin (m.p.=252°C).

2', 4, 4', 6'-tetrahydoxychalcone (naringenin chalcone) was synthesized from naringenin by the method of Moustafa and Wong[176], by treatment with 50% KOH followed by acidification and recrystallization from aqueous ethanol.

Methods

Plot Design and Sampling

Seven spring bread wheats were examined in this trial: the cultivar Otane and the lines CRSW1 and CR5.179 were red-grained while the cultivars Veery,Cook,Oxley and Egret were white-grained. Otane is the current major bread wheat grown in Canterbury and Otago, while the other red lines were from the C.R.D. breeding programme*. Veery is of Mexican origin from the CIMMYT programme and the other white cultivars are Australian.

All cultivars were sown in single 1.5m x 3m blocks. Ears were tagged on the day when they became free of their flag leaf which corresponded to 5-6 days before anthesis in all cultivars.

Collections of all cultivars were made at 15, 20, 25 and 30 days after ear emergence. This period was identified in the 1986/87 study as that of maximal PAL activity. In addition, samples of cultivar Otane were collected at 10, 35, 40, 42, 47, 52

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* Although these numbered lines are not, strictly speaking, cultivars they will be referred to as such for the purposes of the present discussion.

and 58 days after tagging in order to obtain a more detailed profile of enzyme activities and phenolics in this cultivar. All sampling was performed as in the previous trial (sec. 4.2.1).

Germinability of Maturing Grain

Germinability was determined for bulked samples of all cultivars when maturing grain was at approx. 40%, 20% and 10% moisture content (15/1/88, 21/1/88 and 28/1/88 respectively). Random samples of 10 ears per cultivar were collected on these three dates and hand-threshed. Three 20 seed samples of each cultivar at each date were placed in petri dishes on single 7 cm disks of Whatman#1 filter paper moistened with 2ml of distilled water. Dishes were placed in a dark cabinet thermostatted at 16°C and scored for sprouting after 5 days[174].

Extraction and Assay of PAL Activity

Extraction and assay were performed as decribed previously in sec. 4.2.1. Duplicate extracts were prepared from each sample.

PAL Data Analysis

ANOVA of the results was performed using the SAS routine Proc GLM[93]. Grain colour and time were designated as fixed effects and cultivar and extract replicated were designated as random effects. Variance components were estimated as follows:

Variance Estimate=(Effect MS-Error MS)/n

where n is the number of replicates within each level of the effect

These estimates were re-expressed as percentages of the total variance.

Extraction and Assay of CHI Activity

CHI was extracted and assayed according to the methods of Boland and Wong[177].

Samples of 10 seeds were ground with an equal weight of PVPP, in liquid N_2 in a mortar and pestle. The powdered seeds were then homogenized for 30 seconds in 4 ml of ice-cold 50mM Tris-HCl pH 7.4 containing 50mM KCN, using an Ultra-Turrax homogenizer. The homogenate was centrifuged at 500G for 2 minutes and the supernatant was further centrifuged at 20,000G for 10 minutes at 4°C. The supernatant was used as a crude enzyme source.

Assays were performed at 30°C in 1ml polystyrene cuvettes. Crude enzyme (usually 50μl) was added to 1ml 50mM Tris-HCl pH 7.4 containing 50 mM KCN. The reaction was initiated by adding 5μl of 1mg/ml 2',4,4',6'-tetrahydroxychalcone in

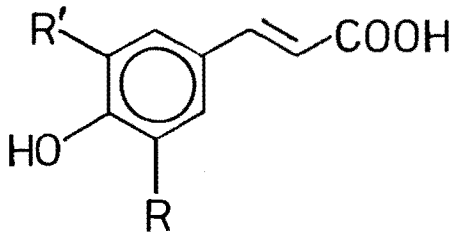
2-ethoxyethanol (final concentration 18.5 μM). Absorbance at 381nm was measured in a spectrophotometer with the cell holder maintained at 30°C. Assays were also run in the absence of enzyme and the increase in the initial rate of disappearance of chalcone in the presence of enzyme was used to estimate CHI activity. Duplicate extracts were prepared from each sample and these were assayed in triplicate. Data was analysed as described above for the PAL data.

Tissue Distribution of Enzyme Activities

The tissue distributions of PAL and CHI activities were initially examined in a sample of cultivar Otane collected 25 days after ear emergence. Grain was dissected into four fractions: outer pericarp; inner pericarp + seedcoat ("green layer"); endosperm + aleurone; embryo. Dissected tissue fractions (other than embryos) were extracted and assayed for CHI activity as described previously. Extraction and assay of PAL activity was also performed as described earlier except that tissue fractions were ground with an equal weight of PVPP and the XAD-2/PVPP column step was omitted. Embryos (usually 10 per sample) were chilled on ice in an Eppendorf tube in 100μl extraction buffer and then crushed with a glass rod. A further 100μl buffer was added and the suspension was further dispersed using a 50W ultrasonic microprobe for two 30 second periods, with cooling on an ice/salt bath. A further 100μl of buffer and approximately 15 mg of PVPP were then added, and the mixture was left to stand on ice for 5-10 minutes. The homogenate was pipetted into a small funnel of Miracloth inserted into the neck of another Eppendorf tube and forced through this by brief centrifugation. The filtrate was centrifuged for 15 minutes at 20,000G at 4°C. The supernatant was then assayed for CHI and PAL activity by the usual methods except that PAL assays were performed in a 600μl final volume.For examination of changes in PAL and CHI activities in tissues over time, embryos were dissected out (after soaking if necessary) and extracted as described above. The de-embryonated seeds were extracted and assayed according to the usual methods.

Phenolics in Developing Grain

All extractions and manipulations were performed in subdued light to minimize isomerization of hydroxycinnamic acids[130] (fig. 4.14). Freeze-dried grains (40-60 depending on dry weight) were powdered dry in a mortar and pestle. The meal was then homogenized for one minute in 20 ml 75% acetone using an Ultra-Turrax homogenizer. The extractant was filtered off using gentle suction through Ford B3 filter paper and the meal was extracted three more times in this manner. The extracted meal was then



R=R'=H *trans*-p-Coumaric Acid
R=CH_3O, R'=H *trans*-Ferulic Acid
R=R'=CH_3O *trans*-Sinapic Acid

Figure 4.13 Major Hydroxycinnamic Acids of Wheat Grain.

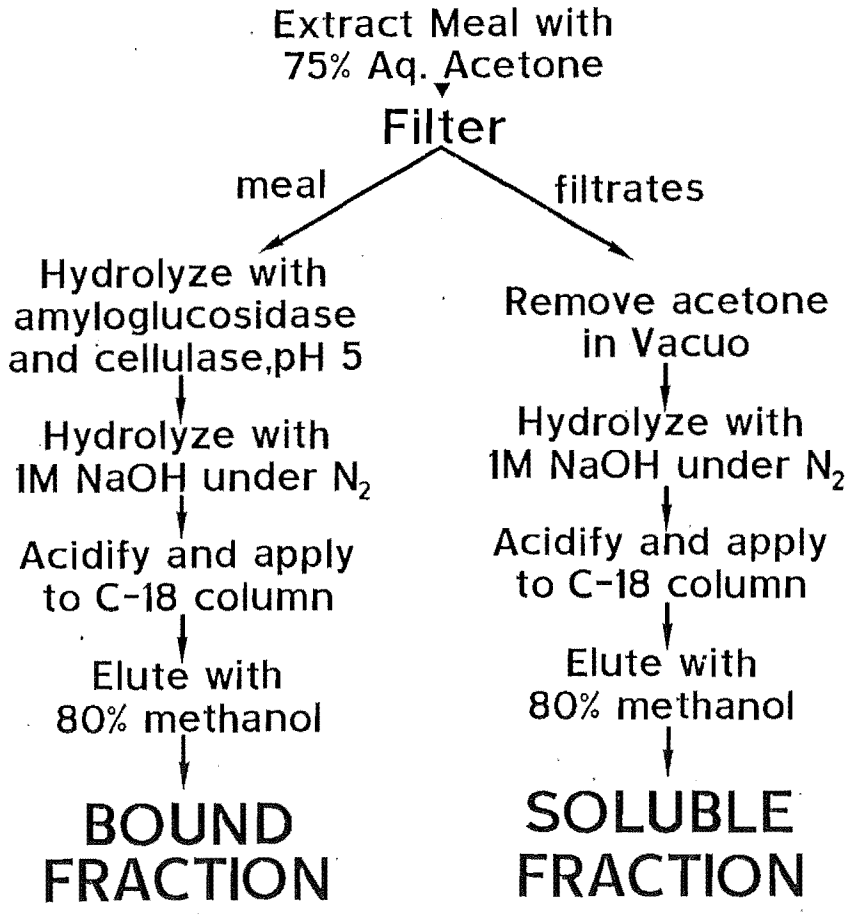


Figure 4.14 Sample Preparation for HPLC of Hydroxycinnamic Acids in Wheat Grain.

washed on the filter with 20 ml acetone and left to dry in a tared dish in the dark. Acetone was removed from the combined filtrates at 30°C *in vacuo* and the volume was adjusted to 20 ml with distilled water.

Aliquots of this extract were assayed for soluble phenolics using Folin-Ciocalteau phenol reagent according to the methods of Swain and Hillis[82]. This reagent has been reported to be less susceptible to interference by reducing agents than the Folin-Denis reagent employed earlier[89].

The aqueous phase was filtered through two layers of Miracloth and 15ml of this was mixed in a stoppered tube with 5 ml 4M NaOH, purged with N_2, sealed and left in the dark for two hours. The hydrolysate was then adjusted to pH 2.5 with 50% TFA, filtered through a Whatman GF/C glass fibre filter and applied to a 10mm x 15mm column containing 250mg of C-18 which had been previously equilibrated with 5mM TFA. Elution was aided by gentle suction. The column was washed with a further 2.5 ml of 5mM TFA and then eluted with 2ml of 80% methanol. The 80% methanol eluate was diluted to 10 ml in a volumetric flask with 0.01M citrate buffer pH 5.4. This is referred to as the "soluble acids" fraction.

The dried extracted meal was powdered in mortar and pestle and 500mg was weighed into a screw-capped centrifuge tube. Sigma Amyloglucosidase (50mg), Novo Celluclast (500μl) and 15 ml 0.05M acetate buffer pH 5 were added and tubes were incubated for 2 hours at 55°C in the dark with occasional shaking. Then 5ml of 4M NaOH were added, the tubes were purged with N_2, sealed, and left in the dark at room temperature for a further 2 hours. Tubes were then centrifuged for 10min at 11,000g.and a 10ml aliquot of the supernatant was diluted with 10ml water and adjusted to pH 2.5 with 50% TCA. This was then adjusted to a volume of 25ml and centrifuged for a further 10min at 11,000G. The supernatant (20ml) was adsorbed onto C-18 as for the soluble fraction. This is referred to as the "bound acids" fraction.

Soluble and bound acid fractions were inspected by TLC on activated silica gel plates developed in benzene:dioxane:acetic acid/90:25:4[133]. Plates were exmined under UV light at 254nm and 365nm before and after exposure to NH_3.

Samples for HPLC were stored refrigerated and in the dark and HPLC was performed within 24 hours of sample preparation. Samples were filtered through a 0.45μm PTFE filter prior to HPLC analysis.

The HPLC system used was an adaptation of that used by Pussayanawin and Wetzel[178] for the analysis of ferulic acid in wheat milling fractions. Separations were performed on a Brownlee 5μm 100mm x 4.5mm RP-18 column fitted and a 15mmx4.5mm guard column mounted in a column heater thermostatted at 30°C. The

mobile phase consisted of 12% methanol in 0.01M citrate buffer pH 5.4 at a flow rate of 1ml/min. Samples were injected through a 50μl injection loop and detection was carried out at 310nm.

Retention times were determined relative to ferulic acid. *Trans*-isomers of the hydroxycinnamic acids were identified by their relative retention times and by spiking samples with authentic standards. Relative retention times of *Cis*-isomers were determined by irradiating authentic standards under UV light (365nm) for one hour followed by re-rechromatography.

4.3.2 Results and Discussion

It was originally planned that four red- and four white-grained cvs would be studied but an unknown rust-susceptible cultivar was inadvertently sown in place of one of the red cultivars. The mature grain of the cultivars used in the study are shown in plates 4.1 and 4.2 before and after soaking in 2M NaOH. This clearly demonstrates the characteristic colours exhibited by red-grained cultivars, the possible chemical basis of which have been discussed in section 3.4.

Growth and Development

Ear emergence and anthesis ocurred earlier than in the 1986/87 trial. This may reflect the effects of sowing in blocks rather than strips, since it was noted that the plots in the earlier trial lagged block planting of the same cultivars in timing of their development. The dates of 50% ear emergence and days from then to anthesis (in brackets) were as follows: Otane 25/11/87 (4); Oxley 25/11/87(6); Cook 23/11/87(6); CRSW1 23/11/87(4); Veery 21/11/87(6); Egret 27/11/87(6); CR5.179 25/11/87(5). All cultivars attained mature grain colour at between 40 and 45 days after ear emergence*. The development of cv Otane is illustrated in plate 4.3.

Due to the use of tagging to define sample age, samples were noticeably less variable in their stage of development than in the previous trial.

The dry weights of mature grain harvested at the end of the study are shown in table 4.8. One-way ANOVA confirmed that the red and white cultivars were not significantly different in their final dry weights

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* Days after ear emergence will henceforth be abbreviated as p.e. (post-emergence).

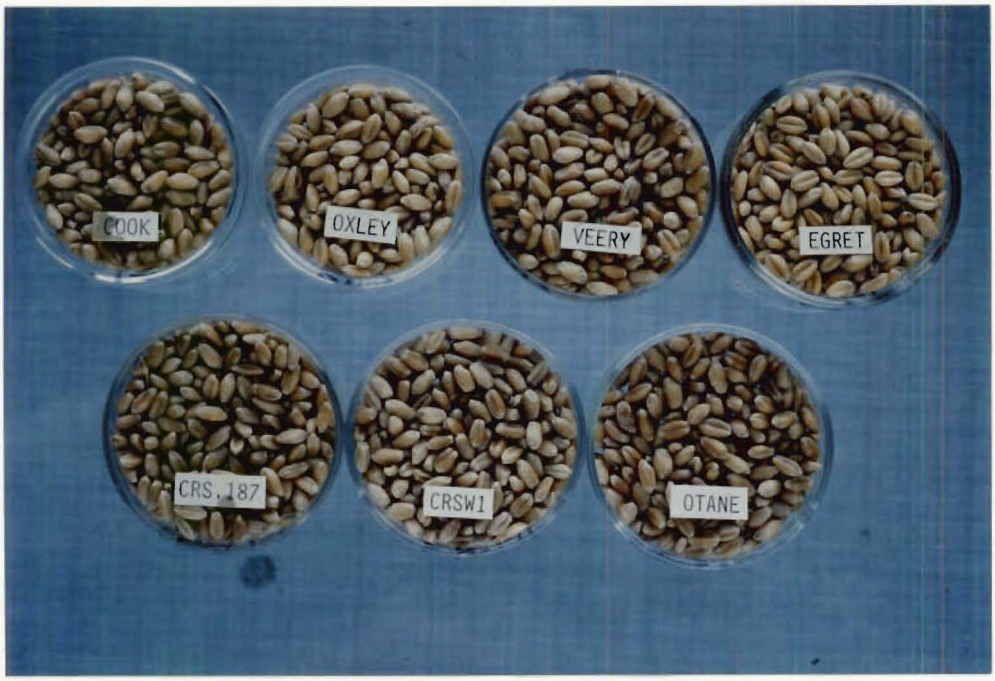

Plate 4.1 Mature Grain of Bread Wheat Cultivars and Lines Examined in 1987/88 Trial.

Plate 4.2 Same Grain Samples in Plate 4.2 Following Soaking for 2 Hours in 2M NaOH.

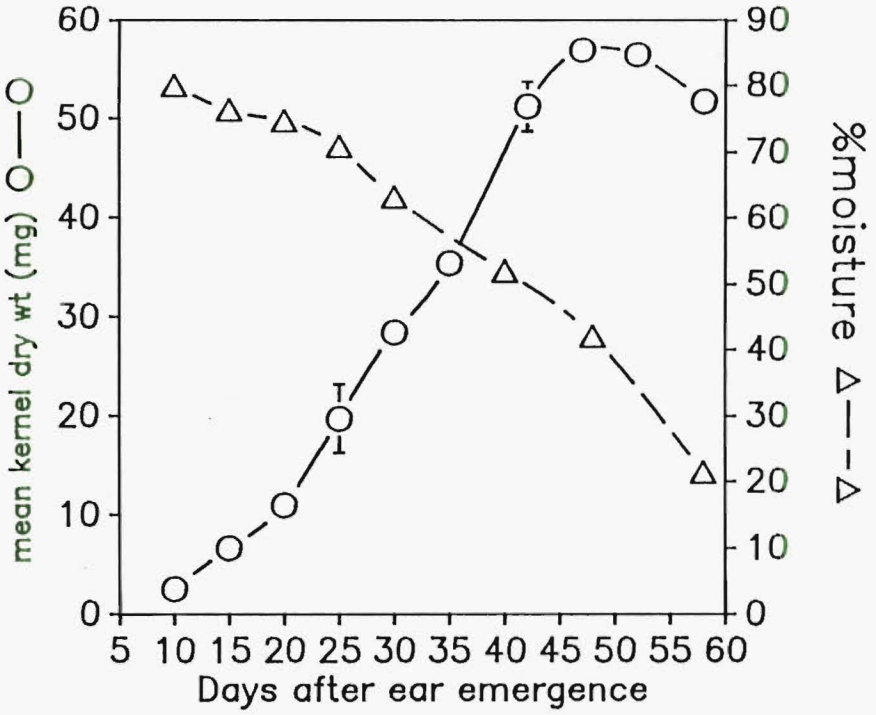

Figure 4.15 Changes in Dry Weight and Moisture Content of Developing Grain of cv Otane in 1987/88 Trial (Points represent mean±sem for n=3 samples).

Plate 4.3 Development of Grain of cv Otane in 1987/88 Trial.

Table 4.8 Dry Weights of Mature Grain from 1987/88 Trial

Cultivar	**Mean Dry* Weight(mg)**	
Veery	47.8	From 1-way ANOVA of mean grain
Otane	47.4	weights (test of null
CRSW1	42.9	hypothesis that red and white
Cook	42.3	cultivars are not significantly
Egret	39.9	different) **P>F=0.67**
CR5.179	39.1	⇒ **Not** significantly different
Oxley	36.1	

(* Determined for samples of 100 seeds.)

Changes in dry weight of cv Otane during grain development are shown in fig. 4.15. Increase in dry weight was linear from 10 to 45 days p.e. The slight drop in the dry weight of cv Otane after 55 days p.e., which was also observed in the 1986/87 trial, has been previously noted by several workers[189] and probably reflects metabolic activity in the mature grain.

Germinability of Maturing Grain

The germinability of grain was examined after mature grain colour had developed and dry weight increase had ceased. This indicated that all the white-grained cultivars exhibited high (80-100%) germinability at harvest ripeness (moisture content 20-12%) under the conditions employed but that of the red cultivars was very low (fig. 4.16). Notably, the germinability of cv Otane was significantly higher than that of the other red cultivars at harvest ripeness. Otane was the major cultivar sown in Canterbury in the 1987/88 season and considerable pre-harvest sprouting was experienced due to warm and wet conditions prevailing in February 1988.

Although simple germination tests such as that employed in this study are widely used in cereal breeding and physiological studies their interpretation demands caution. Lack of germination in such a test can be due to immaturity, dormancy, inviability or lower germinative vigour and therefore interpretation in terms of dormancy alone may be misleading[180]. Germinability may also be influenced by by morphological or inhibitory properties of the bracts when still in the ear[201]. Although inviability was not compensated for in this study the results clearly indicate that there were marked differences in the germinative behaviour of the red and white cultivars used in this study and these could reflect both differences in dormancy and/or the promptness or vigour of germination.

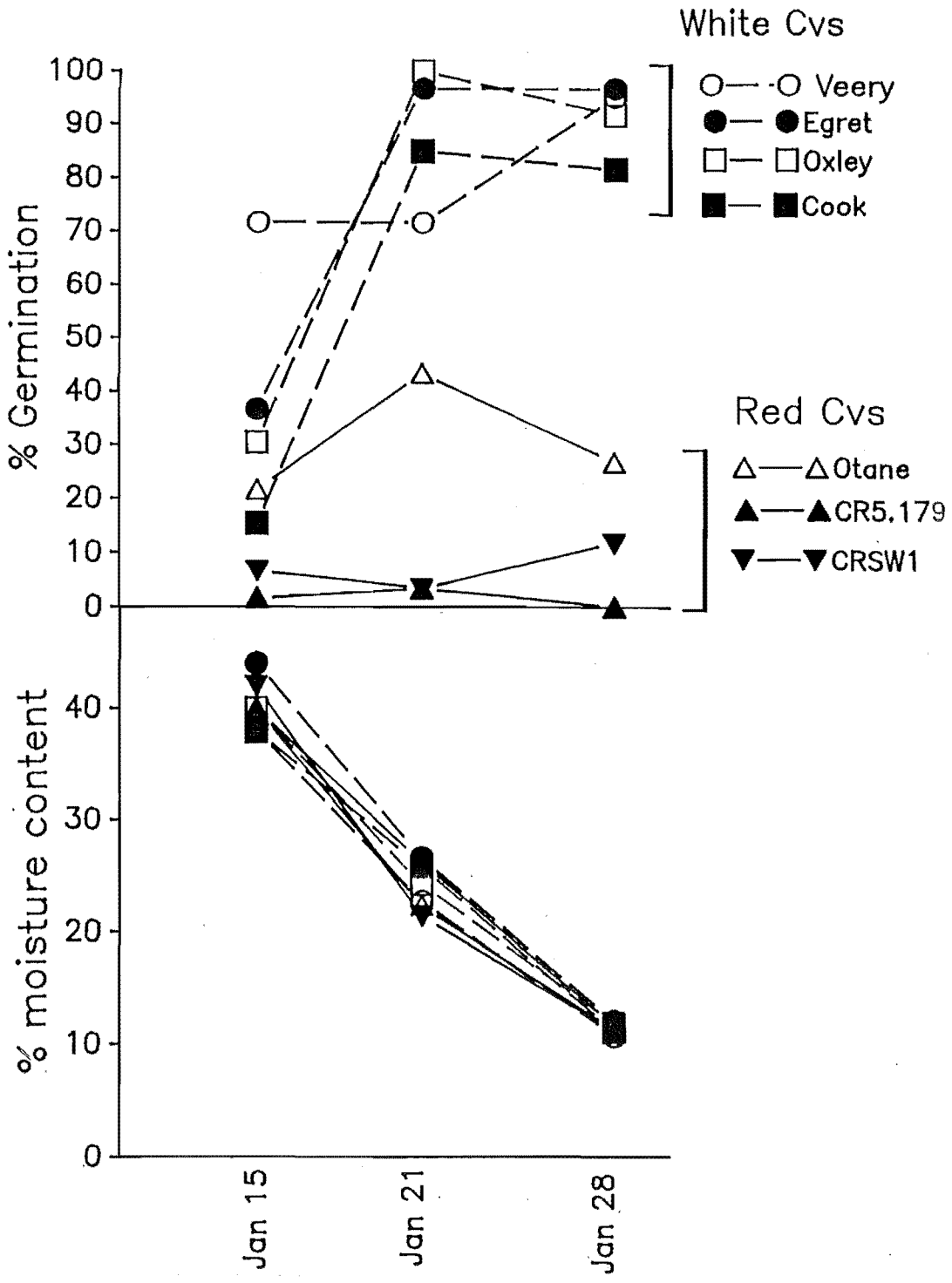

Figure 4.16 Changes in Germinability and Moisture Content of Maturing Grain of Seven Cultivars in 1987/88 Trial (points represent means for n=3 samples).

PAL Activity in Developing Grain of cv Otane

A much more detailed and complete profile of PAL activity was obtained for cv Otane in this trial (fig. 4.17). These results suggested that there is a relatively sharp peak in PAL activity around 20 days p.e., followed by decline to a plateau during the dough stage. Following attainment of mature grain colour at around 45 days p.e., activity declined rapidly to insignificant levels.

The content of soluble phenols per seed exhibited a broader peak similar to that observed in the previous trial, with content at a maximum just prior to devlopment of mature grain colour (fig. 4.17). This profile bore a remarkable similarity to that observed for alkali-labile bound ferulic acid (fig. 4.26).

The greater detail evident in the PAL profile and higher maximal activity observed are most probably due to more precise definition of sample age by tagging rather than differences in activity between the two trials. Although this change in sampling method reduced noise and facilitated comparison of cultivars the change in definition of the actual populations studied would render any quantitative comparisons between the two sets of results meaningless.

This PAL activity profile closely resembles those observed in many other plant systems such as parsley cell suspension cultures[181] following induction of PAL synthesis. A variety of plant materials have yielded fractions that inactivate PAL enzymatically and it has therefore been suggested that this common pattern of activity may arise from interaction between the competing processes of PAL synthesis and inactivation[182]. The existence of such PAL inactivating systems in developing cereal seeds seems likely, as they have been reported from seedlings of rice[184] and barley[185]. Bolwell and co-workers[7] have shown that addition of *trans*-cinnamic acid to elicited bean cell cultures can cause a loss in induced PAL activity by reducing transcription of PAL subunit genes. They have also obtained evidence that *trans*-cinnamic acid can induce synthesis of a proteinaceous PAL inactivator. These observations and other evidence suggest strongly that cinnamic acid, or some metabolite of it, may act as a signal for regulation of phenylpropanoid biosynthesis through effects on both PAL synthesis and inactivation.

While the factors discussed above are likely to be involved in regulation of PAL activity in the developing wheat grain it is important to consider the contribution of individual tissues to total activity of the seed. The cereal seed is a composite organ consisting of diploid parental (testa and pericarp), diploid filial (embryo and scutellum) and triploid filial (aleurone and endosperm) tissues. These genetically and morphologically distinct tissues undergo separate but inter-related programs of development within the developmental program of the whole seed. Therefore phenolic

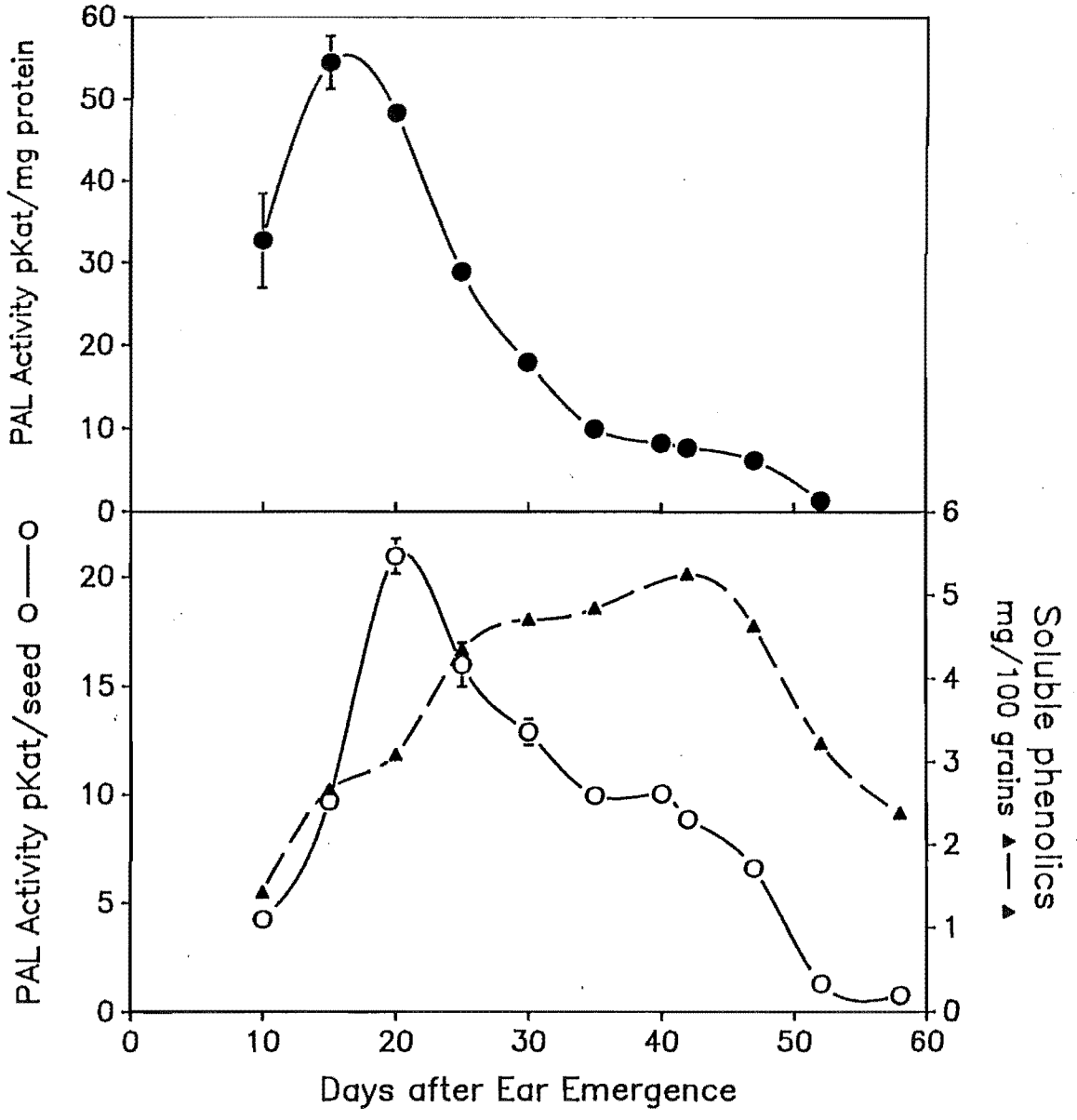

Figure 4.17 Changes in PAL Activity ad Soluble Phenolics During Development of cv Otane in 1987/88 Trial (points represent mean±sem for n=2 extracts assayed in triplicate).

biosynthesis could be expected to proceed in the various tissues at varying rates, at different times and to yield diverse products. The influence of ploidy on regulation of development and germination of the grain is as yet unknown[198].

Distribution of PAL Activity

The distribution of PAL activity was examined initially in a sample of cv Otane collected at 25 days p.e. (table 4.9). This suggested that at this time most activity was associated with the testa and pericarp tissues (referred to by some workers as the "green layer" owing to the presence of chloroplasts in the pericarp during the milk stage). This suggests that the major early peak in PAL activity is associated with phenolic biosynthesis in these parental tissues and, in particular, synthesis of phenolic precursors of pigments in the testa of red wheats. Although the presence in bran of alkylresorcinols[39] and bound phenolic acids[178] is well established there is still a lack of information concerning the flavonoids, aminophenolics and other phenolic pigments that may be present in the testa-pericarp of developing and mature grain.

Table 4.9 Tissue Localization of PAL Activity in cv Otane at 25 Days After Ear Emergence.

Tissue Fraction	Activity per Seed* (pKat/seed)	% Total Activity	Specific Activity (pKat/mg protein)
Outer pericarp	2.70	21.8	23.0
Inner pericarp+testa	5.19	41.9	21.6
Endosperm+aleurone	4.00	32.3	9.40
Embryo+scutellum	0.51	4	28.3

(*Values represent the mean of triplicate determinations.)

Significant PAL activity was also present in the aleurone+endosperm fraction. While it was possible that some of this may represent unavoidable contamination from the testa+pericarp it was unlikely that all of this activity was an artefact of the dissection process. The arabinoxylans of wheat endosperm cell walls contain esterified ferulic acid[128] but otherwise the endosperm contains little if any phenolics. However the associated aleurone layer is not only enriched in wall-bound ferulic acid[129] but also probably contains aminophenols[40] and flavonoids[19]. Studies of endosperm and aleurone cell walls during seed development[129,186] have indicated that strongly autofluorescent wall material is laid down relatively early in endosperm development and this also suggests that phenolic biosynthesis was occuring in these tissues.

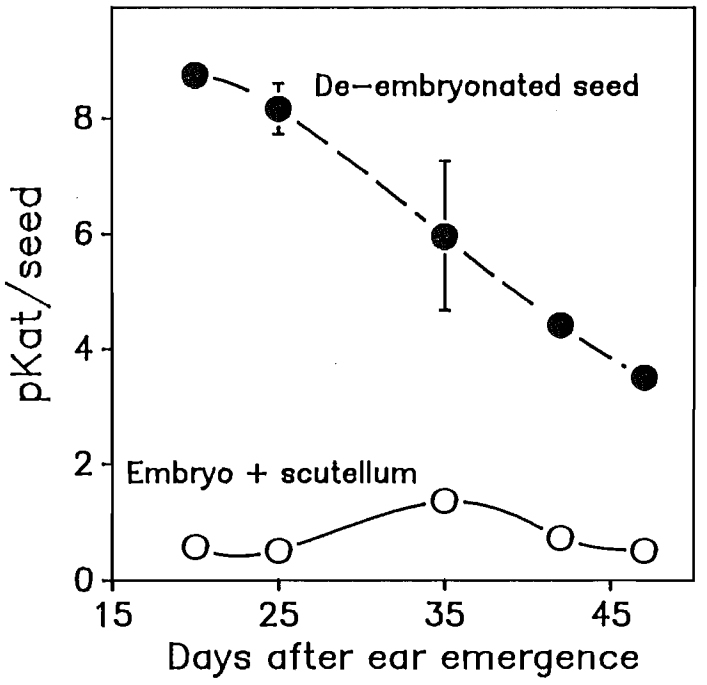

Figure 4.18 Distribution of PAL Activity in Developing Grain of cv Otane (points represent mean±sem of n=2 extracts assayed in duplicate).

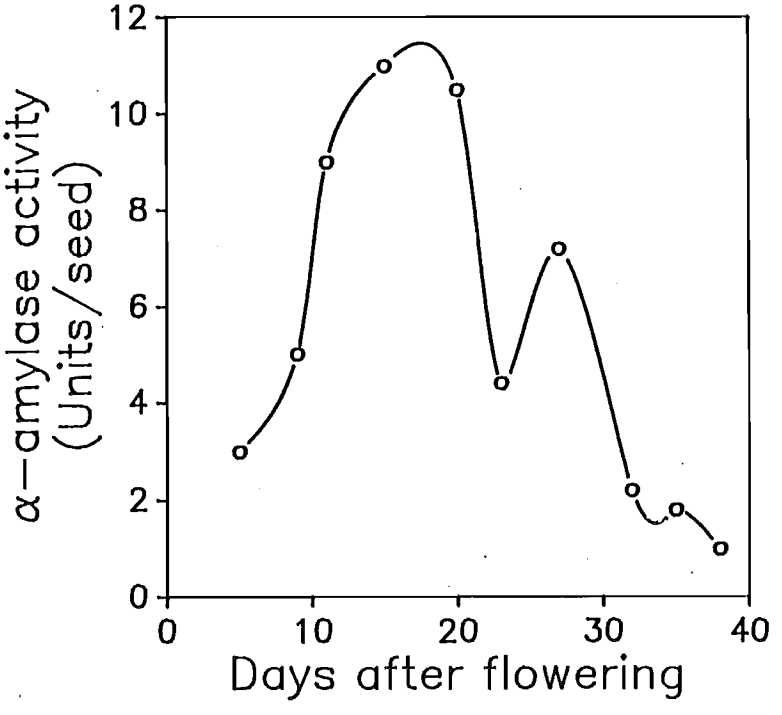

Figure 4.19 Alpha-Amylase in Developing Wheat Grain (From Marchylo et al [171])

The very low level of activity observed in the embryo and scutellum was surprising, since wheat germ contains relatively high concentrations of soluble phenolics. This suggests that phenolic biosynthesis may occur at a later time in these tissues. This possibility was examined by assaying PAL activity in embryo+scutellum and the de-embryonated grain grain at several stages of development (fig.4.18). Unfortunately the material used for this experiment had been stored for over three months and some loss of activity may have occurred. As it cannot be assumed that parallel losses of activity had occurred in all tissues these results on their own cannot be considered conclusive, though the study of CHI activity indicated similar trends (see later in this section). They suggest that PAL activity peaks later in the embryo+scutellum than in the other tissues, in late milky dough stage, and that the shoulder observed in PAL activity in the latter part of grain development may be due in part to activity in these tissues. However it is clear that the major peak of PAL activity observed during grain development was associated with activity in the pericarp+testa.

The pattern of distribution and change of enzyme activity observed in these studies strongly resembles those observed by other workers for α-amylase[171] (fig.4.19) and protease[187] activities in developing wheat grain. These workers also noted that the largest part of the enzyme activities was in the outer layers of the grain and that the decrease in activity in these tissues, as their moisture content decreased, was responsible for the decline in total seed activity during maturation. There is also a decline in nitrogen content in the pericarp+testa during this period[187] and therefore it is likely that enzyme activities could be reduced by non-specific catabolic activities associated with the modification and in some cases destruction of cell structure that these tissues undergo during grain development and maturation[154].

Comparison Between Cultivars of PAL Activities During Early Grain Development

Comparison of PAL activity in seven cultivars from 15 to 30 days p.e. revealed considerably greater differences in activity per seed between red- and white-grained cultivars than those observed in the 1986/87 trial (fig. 4.120). All red cultivars exhibited a marked peak in activity at 20 days p.e. whereas in the white cultivars this was absent or much smaller in the case of cv Cook. There was no apparent relationship between mature grain dry weight and levels of PAL activity in the developing grain. The integral of activity per seed over 15 to 30 days p.e., calculated from areas under a graph of values joined by straight lines, suggested that cultivars fell into three groups (table 4.10). The red cultivars and three of the white cultivars fell into two groups with relatively narrow ranges of activity/seed x time. Both in this trial and the 1986/87 trial cv Cook

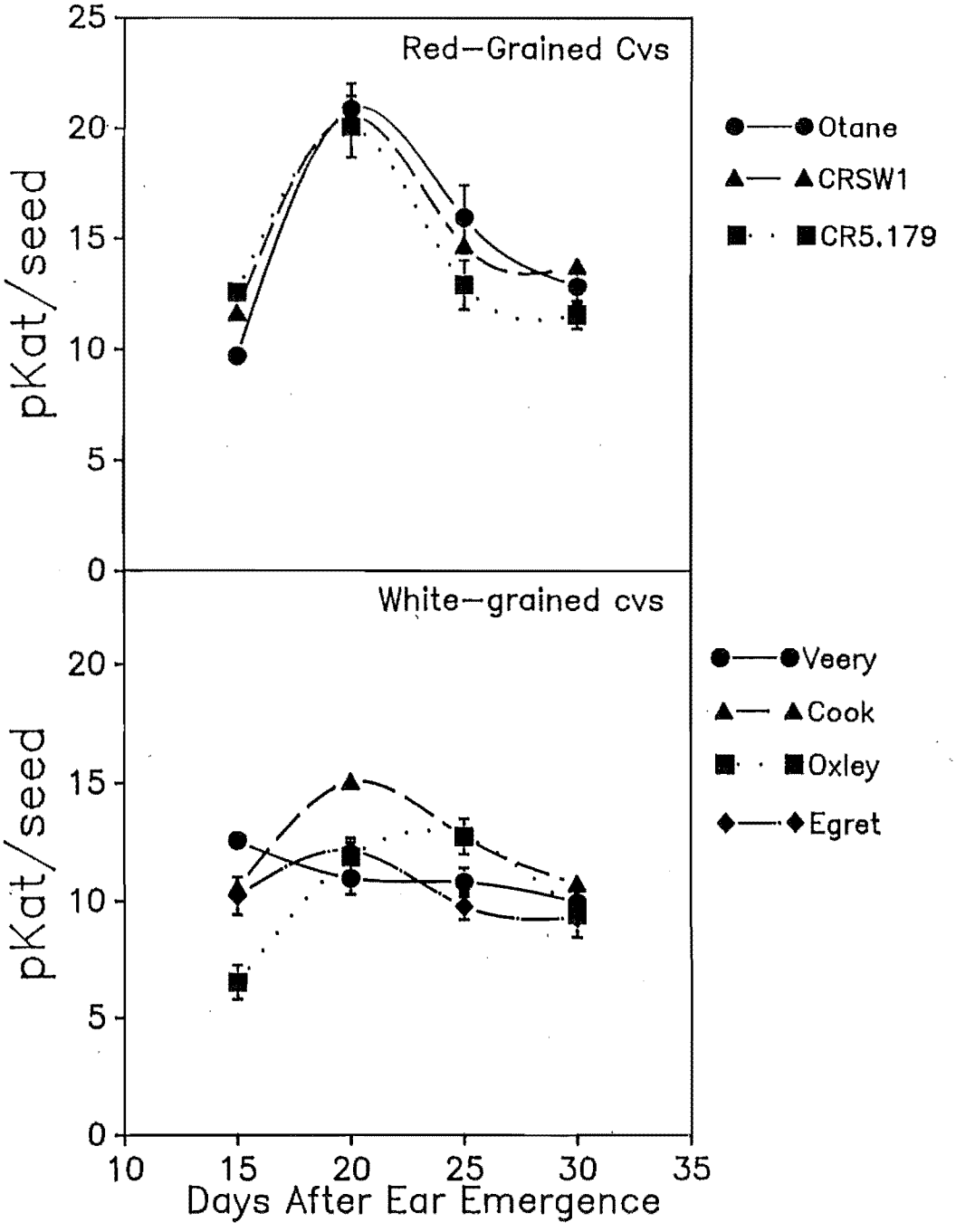

Figure 4.20 PAL Activity During Early Grain Development of Seven Cultivars in 1987/88 Trial (points represent mean±sem of n=2 extracts assayed in triplicate).

appeared to be intermediate in its levels of PAL activity between the red cultivars and other white cultivars examined.

ANOVA of the results was performed using the SAS routine Proc GLM[93], since this permitted analysis of the entire data set, even though the design was unbalanced by loss of one red cultivar. Analysis of the results, as activity per seed, revealed highly significant effects due to grain colour and colour/time interaction, which confirmed that red and white cultivars differed significantly both in their overall levels of PAL activity and in the way this changed over time (table 4.11). The considerable reduction in the variance contribution due to time and the increase in that due to colour compared to the 1986/87 trial indicated that the improved sampling procedures had significantly reduced noise and allowed the differences between red and white cultivars to be more effectively confirmed..

Table 4.10 Integrated PAL Activity Between 15 and 30 Days After Ear Emergence in Grain of Seven Wheat Cultivars in 1987/88 Trial.

Cultivar	**Mature Seed Mean Dry Weight** (mg)	**PAL Activity Integral** (pKat.days.seed^{-1})	
Otane	47.4	295	(The correlation between
CRSW1	42.9	294	PAL integral and mature
CR5.179	39.1	276	kernel dry weight was
			not significant
Cook	42.3	236	r=0.299p>0.05)
Veery	47.8	203	
Oxley	36.1	200	
Egret	39.9	194	

Table 4.11 ANOVA of PAL Activity per Seed Between 15 and 30 Days After Ear Emergence in Grain of Seven Wheat Cultivars in 1987/88 Trial

Source	**DF**	**SS**	**MS**	**F-Value**	**Pr >F**	**%Variance Contribution**
Colour	1	131.42	131.42	39.19	**0.0015**	**34**
Cultivar	5	16.76	3.35	-	-	3
Time	3	189.39	63.13	20.04	0.0001	32
Time x colour	3	56.63	18.88	5.99	**0.0068**	**17**
Cultivar x Time	15	47.26	3.15	-	-	12
Extracts	1	1.60	1.60	4.50	0.087	0.3
Extracts x colour	1	1.04	1.04	2.92	0.15	0.3
Cultivar x Extracts	5	1.78	0.35	-	-	0.6
Time x Extracts	3	1.57	0.52	2.79	0.076	0.3
Time x Extracts x Colour	3	0.102	0.034	0.18	0.91	0
Cultivar x Time x Extracts	15	2.81	0.187	-	-	1**

** Coefficient of Variation for Determination of Extract Mean Activities=5%

CHI Activity in Developing Grain of cv Otane

CHI activity was detected initially in extracts of grain at the milk dough stage of grain development. The pH dependence of the isomerization was studied over the range pH 7.2-8.2 and this indicated that activity was optimal at around pH 7.4 (fig. 4.21). This is close to the value of pH 7.6 reported for the enzyme from soyabean seed[126]. K_m was estimated from direct linear plots of initial rates detemined over a range of chalcone concentrations. This indicated a K_m of around 13μM which is also similar to the value of 21μM reorted for the soyabean enzyme. Due to the instability of this chalcone it is difficult to determine the purity of a sample and thus the K_m could be considerably lower than this.

CHI activity was determined in the same samples used in the study of PAL. The profile of CHI activity per seed in cv Otane exhibited some similarity to the PAL profile (fig. 4.22). Maximum activity per seed also occurred at around 20 days p.e. and this was followed by a general decline in activity. There was also some suggestion of a shoulder in the profile in the dough stage. However unlike PAL, CHI activity could be detected in grain at harvest ripeness. CHI activity also appeared slightly later than did PAL, as no activity could be detected at 10 days p.e. This is consistent with the later occurrence of CHI in the flavonoid biosynthetic pathway.

Since CHS appears to be the primary control point for flavonoid biosynthesis[125] CHI activity is probably best considered as only a general indicator of pathway activity.

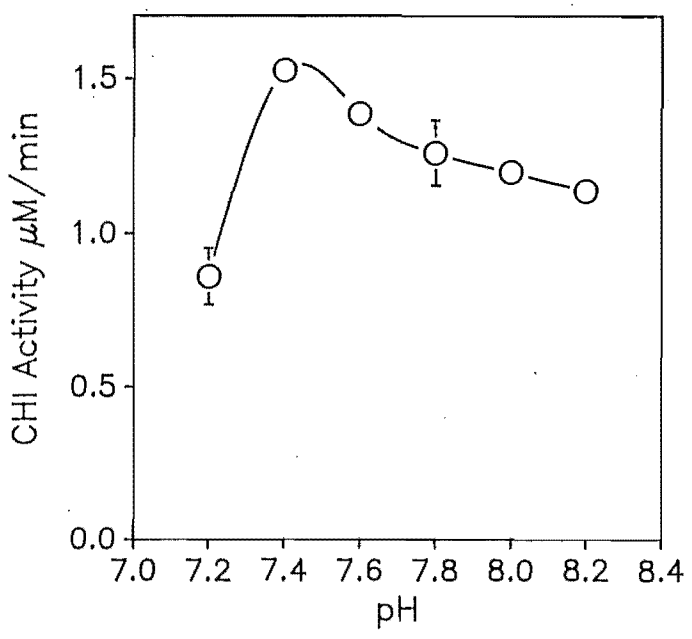

Figure 4.21 pH-Dependance of CHI Activity in Developing Wheat Grain (points represent mean±sem for triplicate assays).

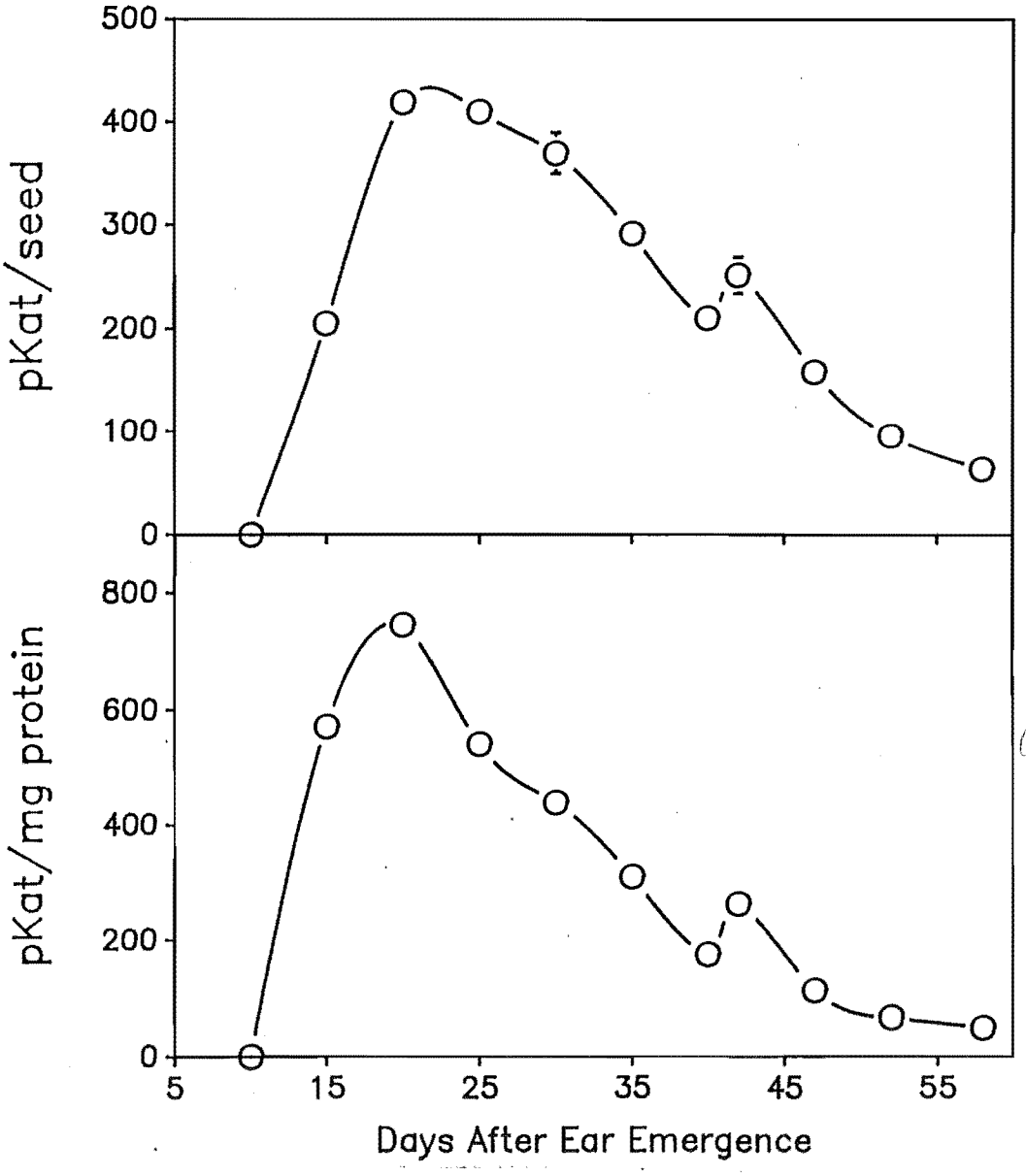

Figure 4.22 CHI Activity in Developing Grain of cv Otane in 1987/88 Trial (points represent mean±sem of n=2 extracts assayed in triplicate).

Distribution of CHI Activity in Developing Grain

The distribution of CHI was initially examined in a sample of cv Otane collected at 25 days p.e.(Table 4.12). This suggested that more than 90% of the activity at this stage was localised in the pericarp and testa layers and, therefore, that the major peak in CHI activity per seed probably represents flavonoid biosynthetic activity in these tissues.

Table 4.12 Localization of CHI Activity in Immature Grain of cv Otane at 25 days After Ear Emergence

Fraction	**Activity** pKat/seed*	**% of Total Activity**
Embryo+Scutellum	7.17±2.0	2.6
Aleurone+Endosperm	13.67±1.5	4.9
Outer Pericarp	45.68±20.8	16.4
Inner Pericarp+ Testa	212.71±25.7	76.2

(* Values represent mean±std. error for n=3 assays)

The traces of activity detected in the aleurone+endosperm fraction may represent inadvertent contamination during dissection, though histochemical evidence from oats[19] suggests that the aleurone layer in cereal grains may contain flavonoids.

The low level of CHI activity observed in embryo+scutellum fraction at 25 days p.e.suggested that flavonoid biosynthesis might occur at a later time in these tissues since they are a rich source of flavonoids in the mature grain[27]. CHI activity was compared in embryo+scutellum and in de-embryonated seed of cv Otane from 20 to 47 days p.e.. This suggested that CHI activity in the embryo+scutellum tissues was at relatively low levels during early grain development but increased to reach a plateau of around 100 pKat/seed from 35 days p.e.(fig. 4.23). Assay of embryos + scutella dissected from mature stored grain following brief soaking showed that these exhibited the same level of activity as those in dissected during the latter stages of grain development. Activity in the de-embryonated seed, representing predominantly pericarp+testa activity, exhibited the same decline with grain dehydration observed earlier for PAL. However these observations are more conclusive than the corresponding study of PAL in dissected grain, due to the apparently greater stability of CHI under the storage conditions employed. The presence of CHI activity in the mature grain suggests that the enzyme may be less subject to specific or non-specific inactivation during grain maturation or storage.

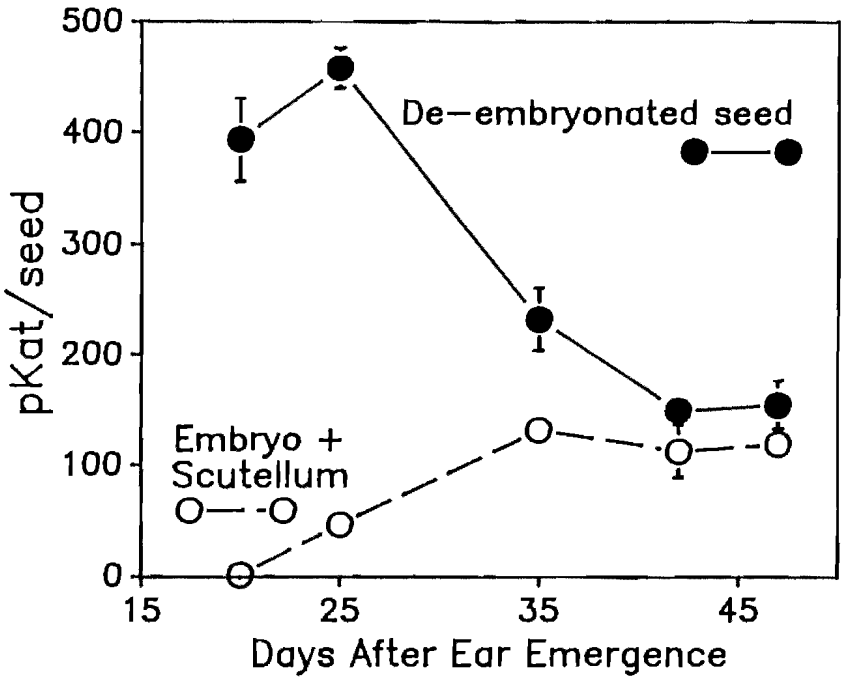

Figure 4.23 Distribution of CHI Activity in Developing Grain of cv Otane in 1987/88 Trial (points represent mean±sem of n=2 extracts assayed in triplicate).

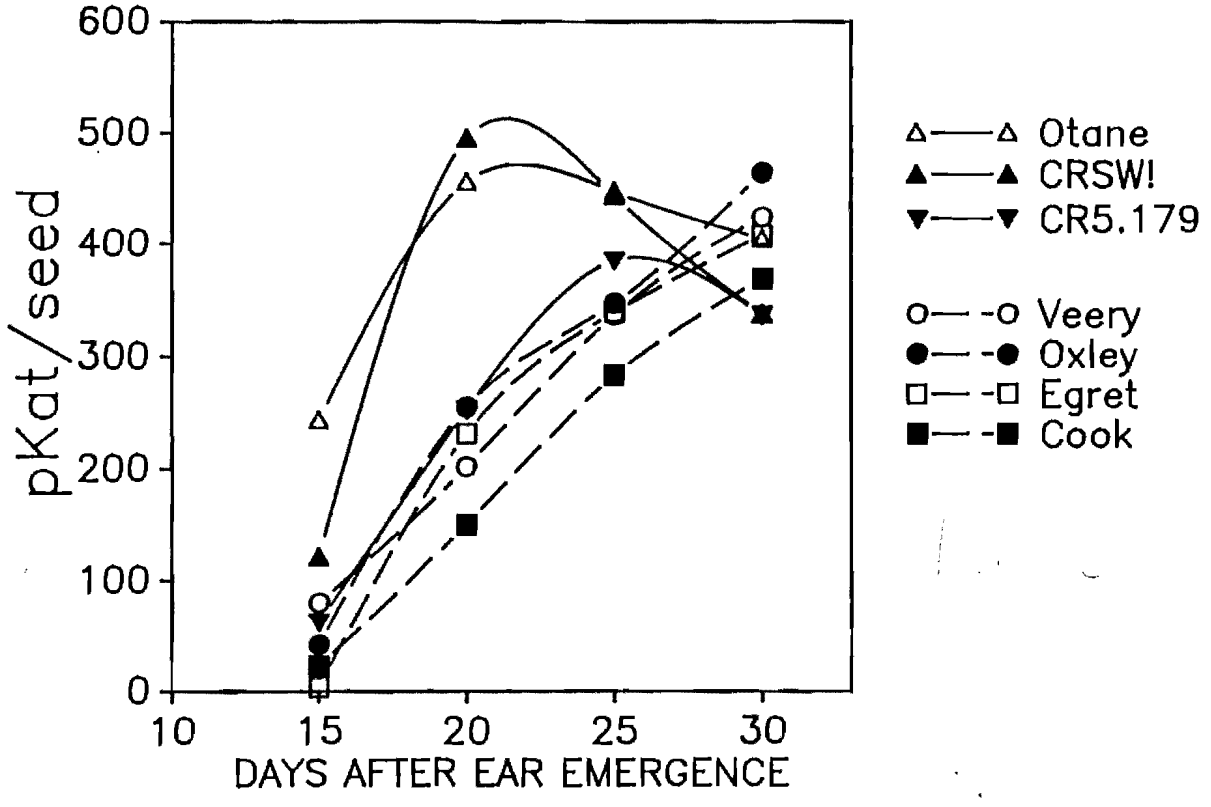

Figure 4.24 CHI Activity During Early Grain Development of Seven Cultivars in 1987/88 Trial (points represent mean of n=2 extracts assayed in triplicate).

Studies of the biosynthesis of catechin and proanthocyanidins in developing barley grain have shown that synthesis is maximal around 12 days after anthesis and that it occurs in the testa[189]. Histochemical studies have confirmed that PAs are localized in the testa of mature barley grain[191]. Since the testa is also the location of pigmentation in the mature wheat grain[154] it is likely that pigment precursors are synthesized during this early phase of flavonoid biosynthesis.

Comparison of CHI Activities in Developing Grain of Seven Cultivars

Comparison of CHI activity per seed in the same samples of the seven cultivars analysed for PAL activity also suggested significant differences between the red- and white-grained cultivars (fig. 4.24). All three red cultivars exhibited a peak in activity per seed at 20-25 days followed by a decline, whereas all the white cultivars showed a steady increase over the period 15-30 days p.e. For the future it would be desirable to investigate the changes in CHI activity and its distribution in a white cultivar.

ANOVA of the results did not reveal a significant effect due to grain colour but a highly significant colour/time interaction effect (table 4.13). This confirmed that over the period 15-30 days p.e.there was a significant difference in the pattern of change in activity over time exhibited by the red and white cultivars.

Table 4.13 Results from Repeated Measures ANOVA of CHI Activity per Seed from 15 to 30 days p.e. for Seven Cultivars†

Source	DF	SS	MS	F	P>F
Colour	1	1060.9	1060.9	5.34	0.069 N.S.
Cultivar	5	992.5	198.5	-	-
Time	3	8506.3	2835.4	72.9	0.0001
Time x Colour	3	1184.5	394.8	10.15	**0.0007**
Cultivar x Time	15	583.6	38.9	-	-

** The coefficient of variation for the assay procedure was 5.8%.
† Only significant effects and their associated error terms have been presented

The results of this comparison and the study of the tissue distribution of activity suggest that the early peak of CHI activity observed in cv Otane could be absent or delayed in the white-grained cultivars. In the absence of a profile and distribution of CHI activity during development of a white cultivar it is difficult to speculate further on the nature of the difference between red and white cultivars. Clearly, the levels of CHI and other enzymes of the flavonoid pathway are only one factor affecting the accumulation of flavonoids in the developing grain. Supply of phenylpropanoid precursors could be a limiting factor and thus the higher levels of PAL activity in red

cultivars could lead to greater accumulation of flavonoids. As well as influencing the synthesis and removal of PAL and other enzymes, it has been shown that *trans*-cinnamic acid can activate CHI *in vitro*[190]. Therefore, in red cultivars containing higher levels of PAL and CHI during early grain development, a synergistic effect on flavonoid accumulation may occur as a result of such a promotion of flavonoid synthesis.

Changes in Ferulic and Other Hydroxycinnamic Acids During Grain Development in cv Otane

Soluble and bound, alkali-labile hydroxycinnamic acids from cv Otane at various stages of development were examined initially by silica gel TLC. This suggested that during early grain development ferulic acid was the predominant hydroxycinnamic acid in soluble fractions but from the milk stage sinapic acid appeared to be present in larger quantities. Sinapic acid is known to occur esterified to C-glycosyl-flavones in the germ[26,27]. By contrast ferulic acid was the major acid in the bound fractions at all stages. Chromatograms of extracts prepared at the late milk stage and thereafter contained a compound which possessed fluorescence and R_f characteristics[133] consistent with its being diferulic acid.

Analysis of extracts by HPLC confirmed and extended these observations (fig 4.25). The HPLC system used was based on a system developed for determination of ferulic acid in milling streams as a measure of bran contamination[178] and this provided rapid and reproducible estimates of ferulic acid in extracts of immature and mature grain with a coefficient of variation for the analysis of approximately 1%. Although optimized for *trans*-ferulic acid, this method also provided useful results for *trans*-p-coumaric and *trans*-sinapic acids, although occasional incomplete resolution from other components present in the later stages of grain development made these considerably more variable.

The quantities of *cis*-ferulic acid observed were generally in the order of 0.5-4% of that of *trans*-ferulic acid. Precautions were taken to avoid exposure of samples to UV light during sample preparation and handling but it is possible that sufficient exposure occurred to account for these levels of the *cis* isomer. Hartley[231] has reported considerably higher proportions (30-40%) of bound *cis*-ferulic acid in hydrolyzates of wheat bran cell walls. The apparent content of bound *cis*-ferulic acid per seed was correlated highly with that of *trans*-ferulic acid ($r=0.92$, $p<0.0001$). However this was not the case for soluble fractions ($r=0.29$, $p=0.07$; see fig. 4.26) and it is likely that this could reflect incomplete resolution in some samples due to relatively low levels of the *cis*-isomer coupled with the presence of other UV-absorbing components with similar

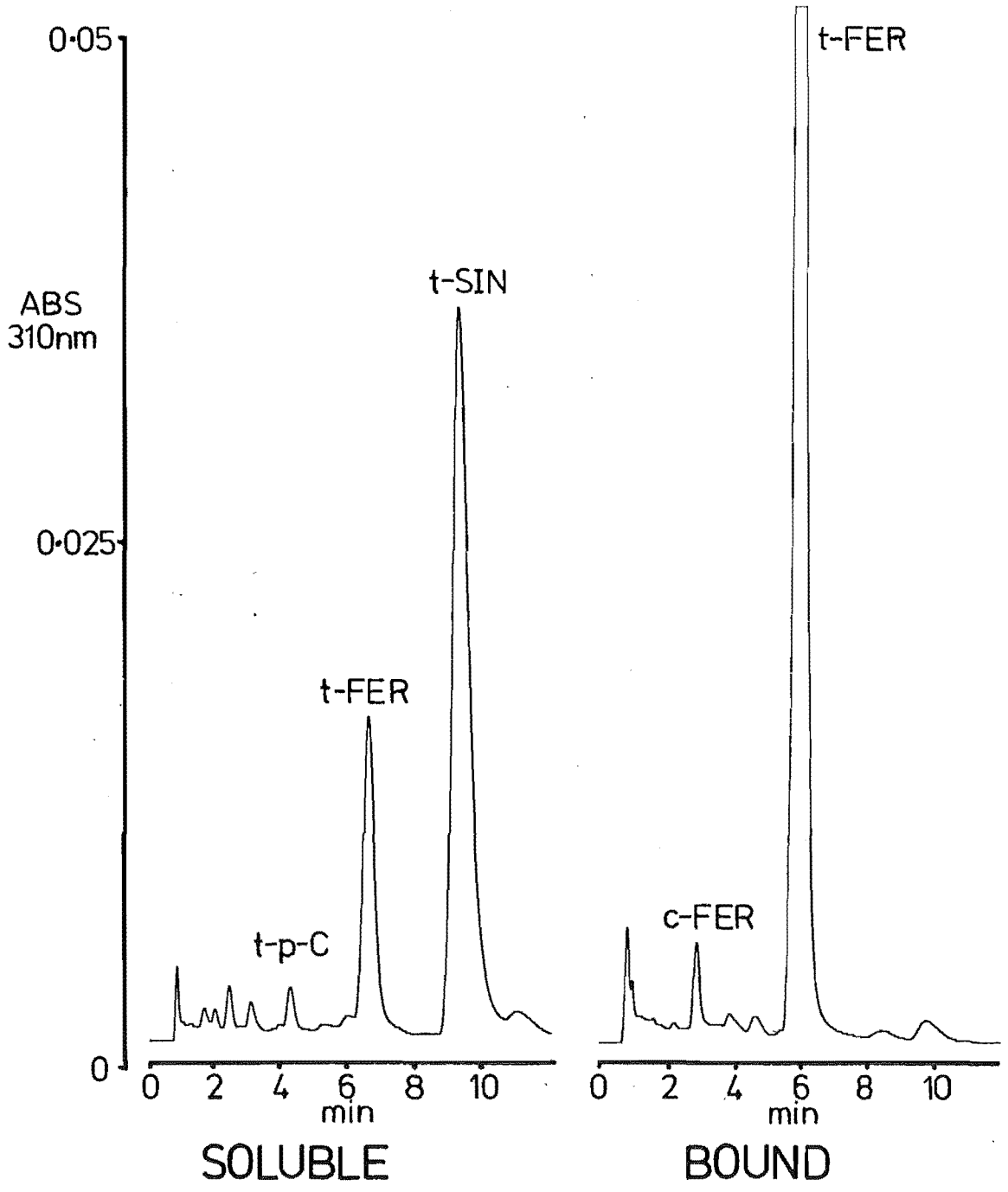

Figure 4.25 HPLC Chromatograms of Hydroxycinnamic Acids in Extracts of cv Otane at Milk Stage (35 days p.e.). [t-p-C = *trans*-p-coumaric acid; t-fer = *trans*-ferulic acid; c-Fer = *cis*-ferulic acid; Sin = *trans*-sinapic acid]

retention times. However it is also possible that the marked increase in the relative amounts of *cis*-ferulic acid in the latter part of grain development could reflect changes in the transparency of the bracts toward UV light. The parallel loss of chlorophyll and dessication in the bracts and pericarp during this stage could result in greater exposure of soluble hydroxycinnamates to UV with a resulting shift in the equilibrium toward the *cis*-isomers.

The changes in *trans*-ferulic acid per seed in cv Otane are shown in figure 4.26. The profile of soluble *trans*-ferulic acid suggests that there are two major peaks in early and late milk stage followed by a decline on maturation. This could represent two independent pools of ferulic acid associated with synthesis in different tissues. Earlier observations on the distribution of PAL and CHI activity suggest that the maximum activity of these enzymes in pericarp+testa and embryo+scutellum coincide approximately with the early and late peaks of soluble ferulic acid content.

By contrast to the soluble fraction, bound *trans*-ferulic acid per grain increased steadily until the grain had attained maximum dry weight. During the final stages of grain dessication and maturation the amount of bound ferulic acid per grain declined by about 50%. The initial increase in bound ferulic acid content showed a high and significant correlation with grain dry weight (fig. 4.26). Presumably this reflects the fact that ferulic acid occurs esterified to cell wall material of bran[178] and endosperm[128] (and probably most other tissues in the grain) and that, throughout most of the phase of grain growth, grain filling is correlated with increase in cell wall material. In addition, ferulic acid may occur esterified to endosperm proteins as it does in barley[146]. This correlation between dry weight increase and bound ferulic acid content suggest also that esterification of ferulic acid to cell wall macromolecules occurs at the same time as cell wall synthesis. When changes in bound ferulic acid were plotted on a dry weight basis (fig. 4.27) a markedly different pattern was observed. The general decline observed in this graph may reflect the lower concentration of bound ferulic acid in endosperm compared to the outer layers of the grain[178,194,193].

Changes in soluble phenolic acids during development and storage of barley grain have been studied by Slominski[192]. He noted similar high concentrations of these acids at the milk and dough stages of grain development as well as a significant decline in soluble ferulic acid on storage. Salomonsson[193] examined changes in bound ferulic and *p*-coumaric acids during barley grain devlopment and reported a similar increase in bound ferulic acid per grain during development. Due to the poor description of grain devlopment and sampling methods and their use of a relatively small number of samples it is difficult to relate the data of these workers to events in grain devlopment and

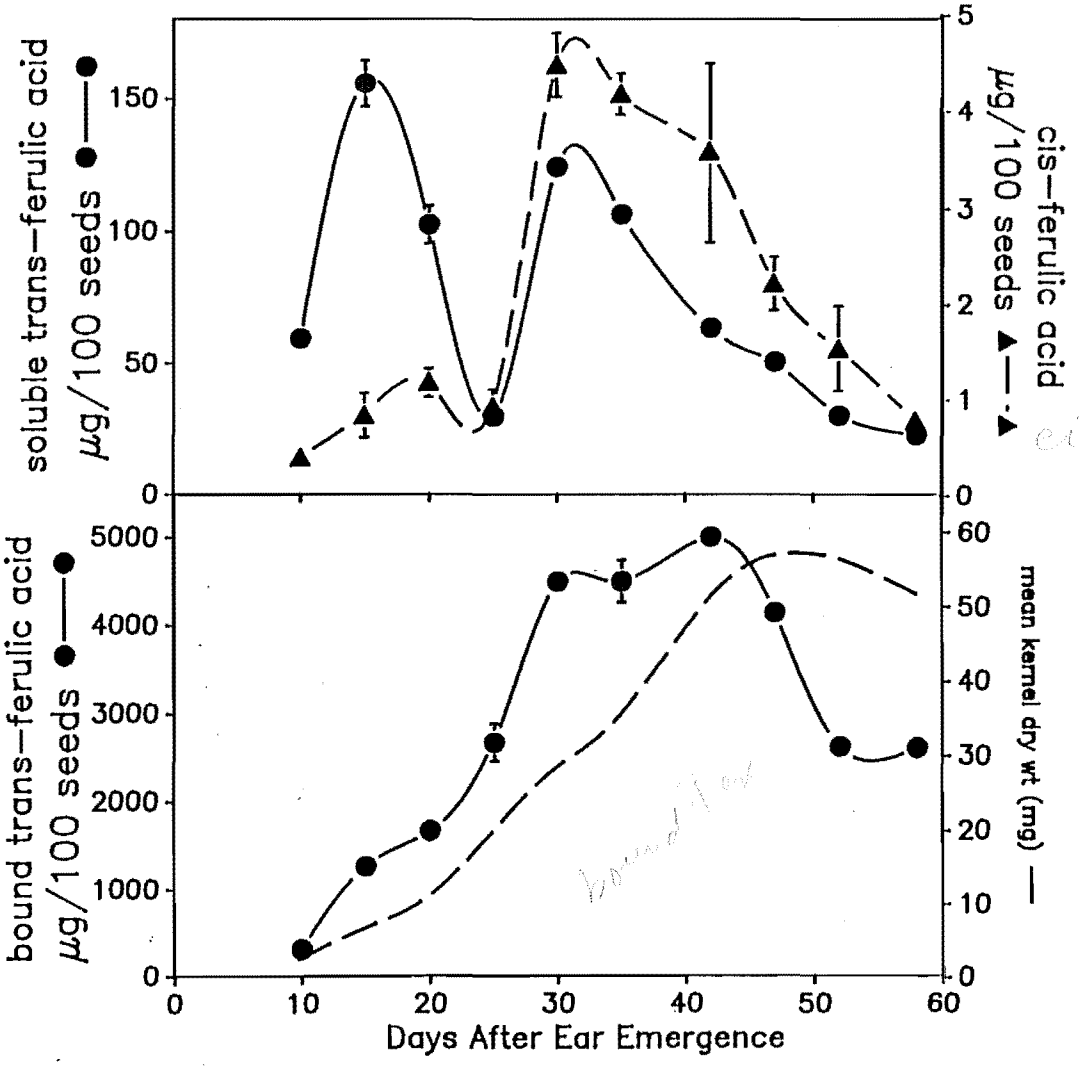

Figure 4.26 Changes in Soluble(top) and Bound (bottom) Ferulic Acid Content of Developing Grain of cv Otane in 1987/88 Trial (points represent mean±sem for n=2 extracts analysed in duplicate)

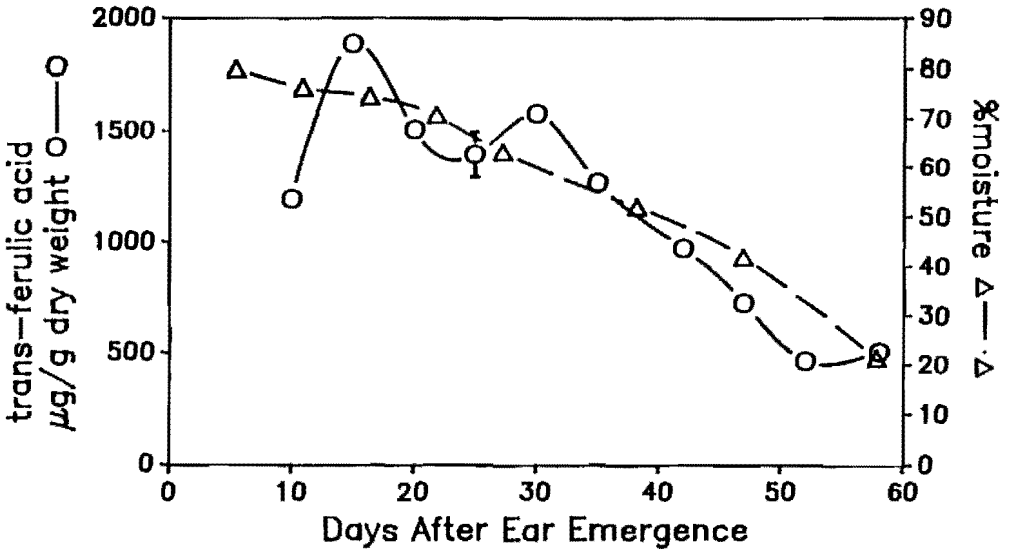

Figure 4.27 Changes in Concentration of Bound *trans*-Ferulic Acid During Development of cv Otane in 1987/88 Trial (points represent mean±sem for n=2 extracts analysed in duplicate)

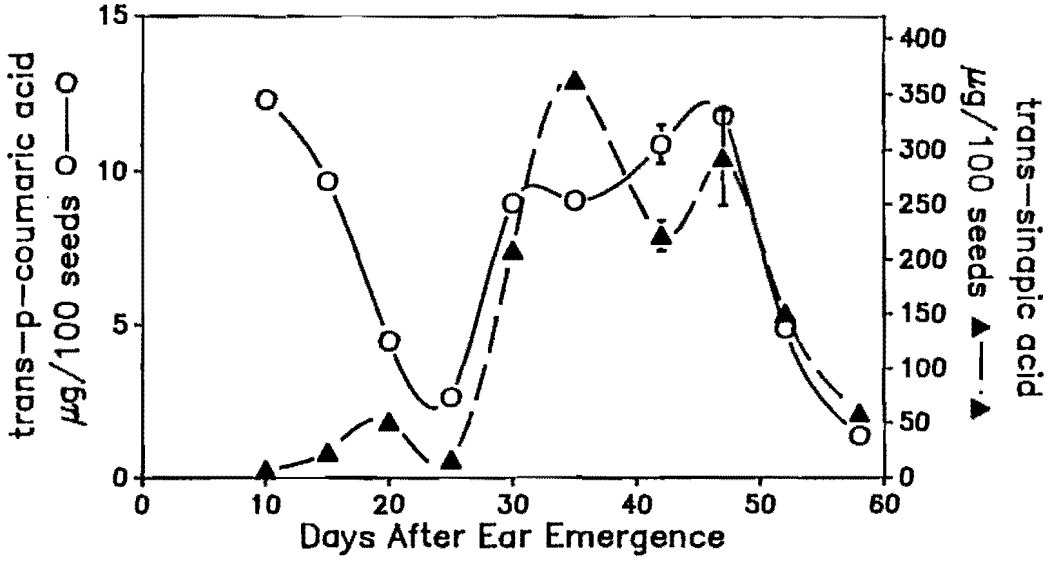

Figure 4.28 Changes in Content of Soluble *trans*-Sinapic and *trans*-p-Coumaric Acids During Development of cv Otane in 1987/88 Trial (points represent mean±sem for n=2 extracts analysed in duplicate)

maturation. Further comparison is also limited by the fact that the barley grain has a husk that is relatively rich in bound phenolic acids[194].

Changes in soluble *trans*-sinapic and *trans*-*p*-coumaric acid exhibited many similarities to those observed for *trans*-ferulic acid (fig. 4.28). Changes in bound *trans*-*p*-coumaric acid exhibited a markedly different pattern of change to that of *trans*-ferulic (fig. 4.29).

Comparison of Ferulic Acid Content of Immature and Mature Grain of Seven Cvs

Soluble and bound *trans*-ferulic acid was assayed in samples of the seven cultivars collected at 20 days p.e. and in samples of mature grain that had been stored at ambient temperature in sealed tins for five months (table 4.14). Although the amount of soluble ferulic acid per grain in cv Otane observed after this period of storage was comparable with that measured in grain at harvest ripeness, the bound ferulic acid had declined by about 50%.

The data obtained from HPLC analysis of these two sets of samples was combined and the data for soluble and bound ferulic acid were each subjected to repeated measures ANOVAs using the SAS routine Proc GLM[93]. The results thus obtained from the test of the hypothesis that content per seed or concentration of ferulic acid was the same in red and white cultivars were as follows:

Variable	**F**	**P>F**
Bound *trans*-Ferulic Acid/100 grains	3.21	0.13
Bound *trans*-Ferulic Acid/g dry wt.	0.90	0.39
Soluble *trans*-Ferulic Acid/100 grains	3.13	0.14
Soluble *trans*-Ferulic Acid/g dry wt.	0.36	0.58

This suggested that although there were significant differences between cultivars (table 4.14), overall there was no significant difference between the groups of red and white cultivars at these two dates.

Although red and white wheats did not differ significantly in their total content per grain or concentration of ferulic acid an interesting trend was noted when bound ferulic acid content per grain was plotted against mature grain dry weights (fig. 4.30). There was not a significant correlation between bound ferulic acid content at maturity and kernel dry weight ($r=0.255$, N.S.) but a significant correlation between the content at 20 days p.e. and the mature kernel dry weight ($r=0.762$, $p<0.05$). However separation of red and white cultivars significantly increased their respective correlation coefficients (red $r=0.968$, $p<0.001$; white $r=0.984$, $p<0.001$). This suggests that there is a different relationship between bound phenolic content in early development, when cell wall

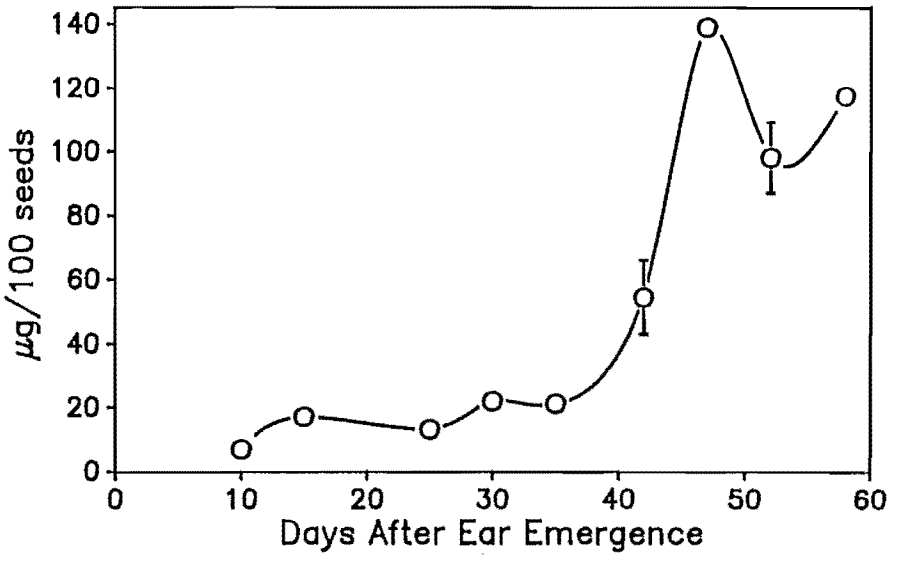

Figure 4.29 Changes in Content of Bound *trans*-p-Coumaric Acid Acid During Development of cv Otane in 1987/88 Trial (points represent mean±sem for n=2 extracts analysed in duplicate)

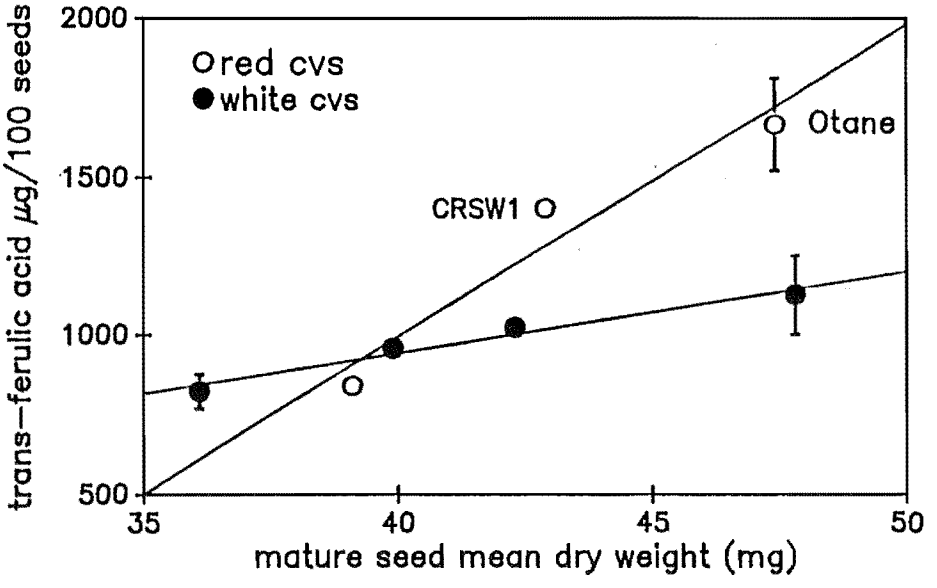

Figure 4.30 Content of Bound *trans*-Ferulic Acid at 20 Days After Ear Emergence and Mature Grain Mean Dry weight for Seven Cultivars in 1987/88 Trial (points represent mean±sem for n=2 extracts analysed in duplicate)

structure is being established, and the eventual dry weight of the grain in red and white wheats.

Table 4.14 Concentration and Total Content of *trans*-Ferulic Acid in Grain of Seven Wheat Cultivars at 20 days After Ear Emergence and in Mature Stored Grain

Cultivar	20 Days p.e.				Mature Stored			
	Soluble μg/100 seeds	Bound μg/100 seeds	Soluble μg/g dry wt.	Bound μg/g dry wt.	Soluble μg/100 seeds	Bound μg/100 seeds	Soluble μg/g dry wt.	Bound μg/g dry wt.
Otane	102.5 *b*	1668 *a*	93.7 *b*	1510 *a*	27.9 *a*	967 *a*	5.68 *bc*	349 *b*
CRSW1	90.7 *d*	1402 *b*	78.9 *c*	1219 *c*	23.9 *bc*	603 *e*	5.41 *c*	238 *g*
CR5.179	94.8 *c*	838 *f*	95.6 *b*	1524 *a*	22.2 *d*	883 *b*	5.68 *bc*	392 *a*
Cook	51.5 *f*	1023 *d*	63.3 *d*	1256 *b*	24.9 *b*	755 *c*	5.91 *b*	313 *d*
Veery	35.8 *g*	1125 *c*	31.4 *e*	976 *e*	27.9 *a*	672 *d*	5.78 *b*	243 *f*
Egret	106.4 *a*	958 *e*	121 *a*	1091 *d*	23.2 *cd*	770 *c*	5.74 *b*	334 *c*
Oxley	62.1 *e*	821 *f*	65.3 *d*	1519 *a*	27.8 *a*	627 *e*	6.97 *a*	274 *e*

**Values followed by the same letter are not significantly different by a Bonferroni pairwise comparison of means at a 5% significance level.

4.4 Conclusion

The work reported here represents the first general study of phenolic biosynthesis in developing wheat grain. Not only has a relatively detailed picture been obtained of the changes in activity of two enzymes involved in phenolic biosynthesis and the content of a major phenylpropanoid during grain development, but these parameters have been compared for a relatively large set of cultivars for a study of this kind. Notably, evidence has been obtained for differences between red and white-grained wheat cultivars in phenolic biosynthesis during early grain development.

The results of the two successive trials highlight some of the requirements for conducting more useful biochemical or physiological studies of cereal seed development. A major criticism of many investigations is that sampling procedures have been inadequately described. The present results suggest that sampling procedure and frequency may have a marked influence on the qualitative and quantitative nature of changes observed in the parameters of interest. While the use of bulked samples may be adequate where larger samples are required or events later in development are being studied, these may obscure important changes during early development when there is significantly more variation within and between individual ears in their degree of development. Most comparative studies have tended to use relatively small numbers of cultivars, which greatly reduces the likelihood of statistically detecting any differences that may exist between them. It has been common practice to report results from investigations of seed development in terms of dry weight or specific activity. While presentation in this manner may be appropriate for comparison of mature grain in relation to end use it is likely to obscure not only patterns of change but also possible differences between cultivars, due to differences in their extent, timing and rate of dry matter and protein accumulation. It is all too often overlooked that values thus expressed are *derived variables* and may be inappropriate for further statistical analysis. It is probably better to keep protein, dry weight or nitrogen measurements separate and to examine their relationship to the variable(s) of interest using covariance or correlation methods.

Few workers have employed analysis of variance methods to evaluate the results obtained from studies of biochemical or other variables during cereal development. Repeated-measures analysis of variance has several advantages over regression techniques when evaluating differences between cultivars concerning a single variable over time. Notably, they allow *a priori* design of tests without the need for the assumptions

associated with curve-fitting and with appropriate design can also provide more information on variability within cultivars due to environmental and other factors.

The activity profiles and distribution of the two enzymes studied bore striking similarities to those observed by other workers for α-amylase[171,172] and protease[187] activities in developing wheat grain. Notably, activities of both PAL and CHI were highest during early grain development and at this time most activity was located in the testa-pericarp. The flavonoid content of wheat bran and its contribution to the pigmentation and other properties of bran is not yet well understood and therefore the existence of high levels of flavonoid biosynthetic enzymes in these tissues is of particular interest. Gordon's study of developing wheat grain[92], as well as studies of barley[189] and sorghum[116], suggest that flavanols and PAs are synthesized during this period. Although the presence of traces of catechin and proanthocyanidins in wheat bran has been confirmed (see sec. 3.3.3) there is no conclusive evidence that these are the principal precursors of the testa pigments of red wheats. It is possible that the relatively low levels of soluble flavonoids in wheat bran compared to seed coats of other cereals is due to its relatively high content of oxidative enzymes[70,71,72]. Phytochemical studies of flavonoids and other phenolics present in the testa+pericarp of the immature grain should allow these questions to be resolved or at least discussed in less speculative terms. Modern techniques of mass spectroscopy and in particular the combination of HPLC and mass spectroscopy, should permit structural elucidation and quantitative analysis of phenolics in the relatively small samples usually available in studies of seed development.

A variety of factors could be involved in the regulation of PAL and CHI activities and phenolic biosynthesis in the developing grain. During the course of grain development the testa and pericarp tissues undergo a unique programme of development that not only involves biosynthesis and growth but also modification, crushing and active dehydration of certain tissues. The appearance of hydrolases and enzymes of phenolic biosynthesis may reflect a post-fertilization trend in these paternal tissues towards development as a protective layer, with a consequent requirement for catabolic activities and the synthesis of phenolics both as protective agents and to contribute toward the physical properties of the seed coat. While it is possible that phenolic biosynthesis in these tissues may be regulated by *trans*-cinnamic acid and other factors as suggested by recent *in vitro* studies[123] it is also likely that enzyme activity and phenolic synthesis is limited by catabolic processes such as dehydration and proteolytic activity. Margna and other workers[123] have argued that phenylpropanoid biosynthesis is usually limited by the supply and availability of L-phenylalanine rather than PAL activity. Recent studies of phytochromeinduced phenolic biosynthesis in radish cotyledons has also provided

evidence that factors other than PAL and CHS activities may limit flavonoid accumulation[210].

Developing seeds are rich sources of plant hormones and it is likely that these are involved in regulation of development, dormancy and germination processes[202,211]. The role of hormones in the regulation of phenolic biosynthesis is, as yet, unclear, although studies of tissue and cell cultures have shown that hormone concentrations and ratios may interact in a variety of ways to influence enzyme activity and accumulation of phenolics[120]. Cytokinin content of developing wheat grain has been shown to peak relatively early, around 45 days after anthesis[212]. Laanest and Margna[213] have observed that kinetin causes a marked increase in the incorporation of L-phenylalanine into C-glycosyl-flavones in barley seedlings, which suggests that cytokinins could be involved in promoting the early phase of biosynthetic activity in the testa-pericarp of cereal grains. Other workers have observed that the IAA[207] and peroxidase[205] content of developing wheat grain exhibit peaks shortly before cessation of dry weight increase similar to that observed in this study for soluble phenolics. As suggested by Machackova *et al*[196], interaction between IAA and phenolics may play a role in regulating the oxidation of phenolics and other processes during grain maturation.

Comparison of PAL and CHI activities between cultivars has suggested that there are significant differences between red- and white-grained cultivars in their potential for phenolic biosynthesis. This observation is consistent with the idea that both the colour and commonly associated dormancy of red wheats may be due to their higher potential for phenolic biosynthesis. The observation made in this and other studies, that red wheat cultivars have consistently higher levels of *o*-diphenol oxidase activity at maturity, suggests that in addition they may have higher potential for phenolic metabolism.

Despite differences in their levels of PAL and CHI activity, no major differences in the total content or concentration of phenolics were observed between red and white cultivars. Differences in ferulic acid content might have have been detected if analyses were conducted at several sampling dates rather than only at 20 days p.e. and maturity, since the profiles observed in cv Otane suggest that the content of specific compounds may fluctuate significantly during development. Ideally it would have been desirable to use HPLC to monitor the concentrations of several compounds, representing major phenolic classes, throughout grain development. Unfortunately this proved impractical due to the difficulties involved in resolving and unequivocally identifying the large number of compounds present in chromatograms. Using several separate methods of sample preparation and analysis, each optimized for quantification of only a few

compounds , would appear to be a more appropriate approach for such a study of cereal phenolics.

Most studies of wheat grain phenolics to date have concentrated on the mature grain or its milling products and few have involved any comparison between different cultivars. This present work suggests that levels of phenolics, in particular hydroxycinnamic acids, are considerably higher in the developing grain than at maturity. It is also clear that they are subject to considerable turnover and metabolism during development and maturation.This would be expected to result in significant effects on the chemical and physical properties of the mature grain[19]. There are a variety of routes by which metabolism and degradation of hydroxycinnamic acids could occur, since these compounds occupy a central role both in the synthesis and in the metabolism of plant phenolics[11]. Conjugation and cross-linking reactions are likely to be of particular significance because of their influence on the properties of protein and carbohydrate. Germinating barley seeds are known to form *o*-glucosides when fed labelled hydroxycinamic acids[197] but it is not known whether wheat grain contains these conjugates. Formation of ester, ether, diaryl and cyclobutyl cross-links by hydroxycinnamates may have marked effects on the physical structures of developing and mature cereal cell walls[195]. It has been suggested that bound acids may act as intermediates in lignin synthesis[216].

Peroxidase appears to play an important role in metabolism and degradation of phenolics. It not only catalyses the formation of some types of cross-links such as in diferulate[133] but may also play a central role in degradation reactions of flavonoids and phenolic acids[11]. Machackova *et al*[196] have suggested that degradation of phenolics by peroxidase may be regulated by auxins in the intact plant. Studies of wheat roots have suggested that wall-bound esterase and peroxidase may be important in the turnover of bound phenolics[217]. Peroxidase can catalyse the formation of methoxy-*p*-hydroquinone from vanillic acid[221] and it is therfore possible that it is involved in the biosynthesis of this aglycone *via* ferulic acid in developing wheat grain. There is also evidence to suggest that peroxidases may be involved in some seed dormancy mechanisms. Recent work suggests that development of hard seed coats in a number of species[153] is associated with phenolic metabolism by peroxidase, rather than *o*-diphenol oxidase activity as suggested by earlier workers. Association between dormancy and peroxidase activity both within and between wheat cultivars has been reported by Noll[225] and Gaspar *et al*[226].

There are at least three mechanisms by which phenolics could influence grain germinability. These are interaction between phenolics and hormones, reduction of

digestibility of macromolecules due to phenolic substitution and through effects of phenolic subsitution and cross-linking on the physical properties of the seed coat. These mechanisms, alone or in combination, could account for the association between seedcoat colour and dormancy.

There is evidence that phenolics can interact with hormones in many plant systems, including the regulation of hydrolase secretion in cereal aleurone layers. Sharma *et al*[214] have observed that several phenolics can reverse abscisic acid inhibition of α-amylase production by wheat aleurone *in vitro*. In contrast, proanthocyanidins have been shown to inhibit synthesis of α-amylase in giberellin-treated barley aleurone[215]. A number of *in vitro* studies have shown that exogenous phenolics can affect cereal seedling growth[229,233] and Podstolski and co-workers[232] have observed an inverse relationship between phenolic content and elongation rate in barley seedlings. These observations suggest that endogenous phenolics could have a marked influence on hormone-mediated processes during germination and early seedling growth.

Fulcher[129] has suggested that the presence of ferulic acid esterification may reduce the susceptibility of aleurone cell walls to digestion by hydrolytic enzymes during germination. Studies on the digestibility of grass cell walls by cellulase [218] and artificially substituted plant fibres[219] have confirmed that digestibility is highly correlated with the degree of phenolic acid substitution. The release of bound ferulic acid esters from barley aleurone during germination has been studied in some detail by Gubler and co-workers[222]. However it is still uncertain whether the differences in susceptibility of aleurone and endosperm cell walls to hydrolysis are due to differences in ferulic acid content or to other differences in wall composition.

Whilst the *in vivo* significance of the previous mechanisms is still unclear there is much evidence to suggest that the seed coat plays an important role in determining germinability. Puncturing or removal of the seed coat has long been known as a means of breaking dormancy. In a comparative study of germination of red- and white-grained wheat cultivars, Wellington[220] noted that the modes of rupture of their seed coats were quite different and concluded that differences in germination behaviour were most likely due to differences in the mechanical properties of the seed coat. Differences in seed coat structure have been noted between red and white cultivars[227] and recent histochemical studies by Duffus and co-workers[222] have suggested that phenolics in the seed coat of barley may play an important role in limiting entry of water and oxygen. Complexation of proanthocyanidins or other phenolics with protein, following enzymic oxidation, could contribute to the physical structure and impermeability of the seed coat[92]. A

contribution by phenolic cross-links to the cohesion of bran cell wall components has been suggested by Ring and Selvandran[134].

In conclusion, this study has provided the first reported information concerning changes during wheat grain development for some key enzymes and products of phenolic biosynthesis. Strong evidence has been obtained that red and white-grained wheat cultivars differ significantly during early grain development in levels of enzyme activity associated with phenylpropanoid and flavonoid biosynthesis. Since most activity at this stage of development was associated with the external layers of the grain these observations are consistent with phenolic compounds contributing both to seedcoat pigmentation and germinability of the mature grain, thus bringing about the association between these traits.

SECTION 5-CONCLUDING DISCUSSION

This thesis has examined the participation of wheat grain phenolics in colouration phenomena from the point of view of milling and baking, the phytochemistry of grain phenolics and their biosynthesis in developing grain. In each of these areas evidence has been obtained to support the hypothesis that phenolics contribute to the colouration of wheat grain and wheaten goods.

While these studies have produced useful information they also illustrate our lack of understanding of the chemistry and biosynthesis of minor components in cereal grains and the difficulties associated with phytochemical studies of mature grains.

The mature cereal grain represents a highly specialised composite organ in which tissues have been considerably modified by crushing, dessication and enzyme activities associated with maturation. These modifications create many difficulties for studies of histochemistry, enzymology and phytochemistry, particularly in the case of labile compounds such as phenolics. These and other studies suggest that mature cereal grains contain a bewildering array of phenolics together with a diverse set of enzymes capable of oxidizing them. In recent years it has become clear that the combination of relatively small amounts of phenolics and oxidases may have a marked effect on the properties of macromolecules. In the future, improved methods for characterising smaller amounts of enzymes and natural products should add greatly to our basic knowledge of these constituents in the mature grain and improve understanding of their practical significance . However, the present investigation suggests that valuable information may be obtained by studies of phenolics and their biosynthesis and metabolism during grain development; this may have several advantages over studies of mature grain. In particular it may facilitate identification of labile phenolics prior to oxidation or other modification during maturation and provide information on regulation of biosynthesis that may be of more direct applicability to future breeding strategies. Studies during development may clarify the role, if any, of the various oxidases in biosynthesis and metabolism of phenolics together with factors involved in their inheritence.

The observations made in this study that phenolic biosynthesis is relatively high in the outer layers of the grain early in development and that they contain proanthocyanidins at maturity highlights our lack of knowledge of bran phenolics, in particular the flavonoids. With the increasing use of cereal bran in bread and other foods a better understanding of these compounds and their role in processing is desirable. The known influence of these tissues on physical and biochemical processes in germination also highlights the need for such information.

Eventually, when a successful procedure is developed for the genetic transformation of cereals, the potential gains will be greatly limited by our understanding of the processes involved in regulation of biosynthesis in the developing grain. For this reason a greater proportion of studies on phenolics and other cereal components will need to be focussed on the developing, rather than on the mature grain.

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Appendix

Table 1 Data From Study of Milling Flour Streams Section 2.2.

Flour Stream	Flour Colour Grade (Kent-Jones Units)	Crumb Colour Grade (Kent-Jones Units)	Crumb Colour Rank (Subjective)	Total Phenols (ug/g dry wt. as gallic acid)	Flavanols (ug/g dry wt. as catechin)	Germ Index (A_{385}nm)	O-diphenol Oxidase Activity (mV/min/g dry wt.)
1st Break	4.4	12.2	7	232±10	43.7±2.2	0.130±0.000	5.90±0.13
2nd Break	3.0	10.7	4	340±2	64.3±0.9	0.145±0.005	6.51±0.09
3rd Break	6.5	13.4	9	373±5	43.7±1.3	0.225±0.00	9.76±0.24
4th Break	12.3	>18	13	577±10	107.3±2.1	0.465±0.005	22.11±0.39
A	-1.6	6.3	1	286±6	70.6±2.9	0.100±0.010	0.39±0.04
B	0.0	8.2	2	229±4	70.0±0.9	0.113±0.003	1.71±0.04
B2	3.7	10.1	-	273±7	24.0±0.7	-	5.38±0.08
C	2.6	10.3	5	229±0	67.9±5.8	0.170±0.010	4.94±0.04
D	3.4	11.0	6	310±7	46.0±1.2	0.193±0.013	5.41±0.04
E	10.1	16.8	11	480±13	97.1±6.3	0.400±0.000	18.73±0.03
F	11.4	>18	12	660±29	80.1±3.5	0.435±0.025	22.96±0.36
G	15.5	>18	14	884±21	129.3±4.4	0.620±0.010	24.66±0.59
H	>18	>18	15	1530±47	122.0±8.4	1.100±0.000	35.46±1.71
X	0.1	8.8	3	201±1	32.6±1.5	0.123±0.008	1.40±0.10
BMR	5.4	12.6	8	410±21	54.1±1.0	0.203±0.003	9.93±0.15
BFD	7.5	15.9	10	324±16	73.3±3.8	0.245±0.015	10.55±0.35

Table 2 Data From Study of Colour and O-diphenol Oxidase in Flours and Wholemeal From 8 N.Z. Wheat Cultivars Section 2.4 (Values represent mean±SEM)

Cultivar	Flour Colour Grade (Kent-Jones Units)	Crumb Colour Grade (Kent-Jones Units)	Flour o-diphenol Oxidase Activity (mV/min/g fresh wt.)	Wholemeal o-diphenol Oxidase Activity (mV/min/g (fresh wt.)
Weka	-0.20±0.00	7.70±0.00	0.79±0.08	3.19±0.10
Rongotea	1.75±0.05	9.95±0.05	1.64±0.17	13.14±0.75
Oroua	1.65±0.05	9.30±0.10	1.04±0.04	10.58±0.55
Otane	0.35±0.05	8.65±0.05	1.26±0.12	14.96±1.55
Kotare	-0.30±0.00	8.25±0.05	1.25±0.05	8.87±0.68
Bounty	-0.10±0.00	9.10±0.10	1.23±0.10	7.16±1.05
Arawa	0.90±0.00	7.90±0.10	0.68±0.05	3.19±0.32
Advantage	0.40±0.00	8.95±0.15	1.30±0.03	6.44±0.80